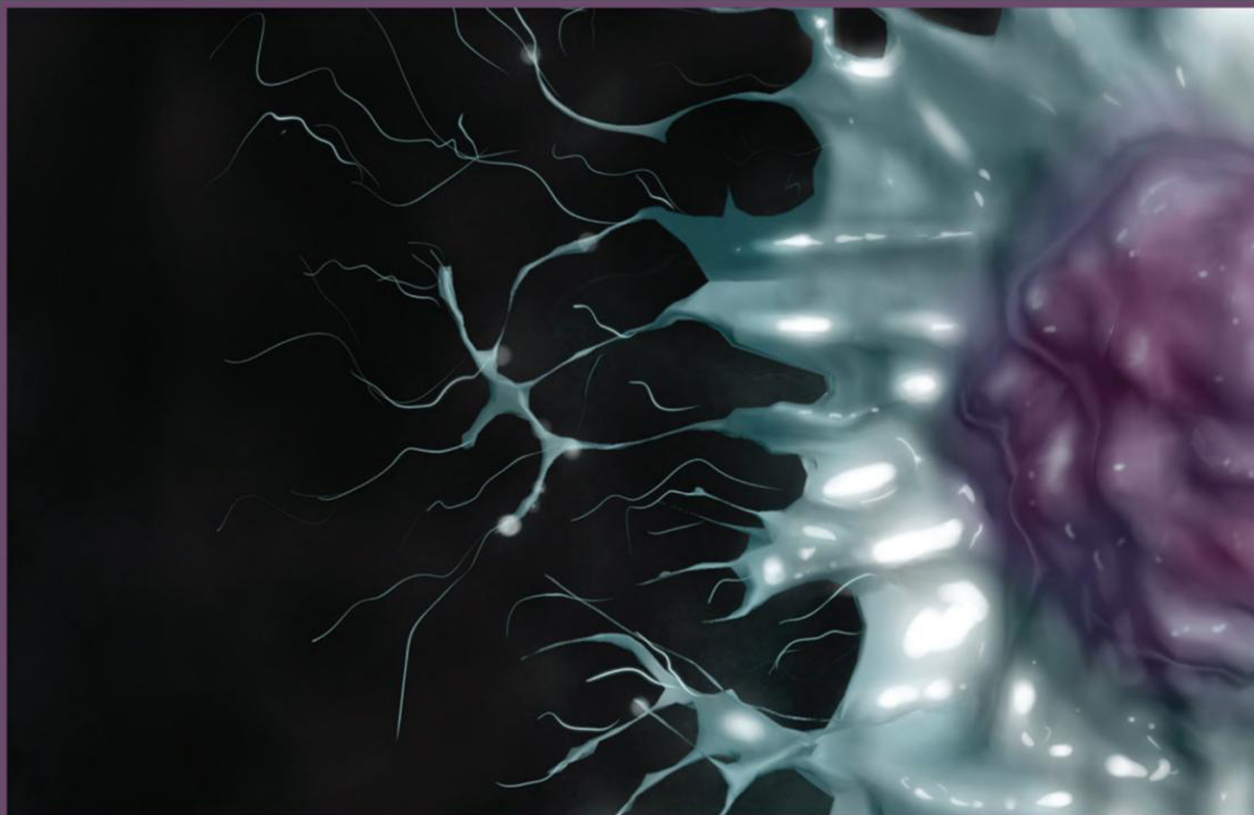


WOODHEAD PUBLISHING SERIES IN BIOMATERIALS



HANDBOOK OF BIOMATERIALS BIOCOMPATIBILITY



Edited by
MASOUD MOZAFARI

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HANDBOOK OF BIOMATERIALS BIOCOMPATIBILITY

Edited by

MASOUD MOZAFARI



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Preface

Over the past decade, I have been working on different classes of biomaterials for applications ranging from advanced therapeutic devices, controlled delivery systems, to tissue engineering, etc. The key point in almost all projects in the field is the response of the host tissue to the biomaterial, known as biocompatibility. Although many groups are working on the cellular and molecular interactions of biomaterials, there is still a lack of precise information regarding the complex mechanisms of biocompatibility. These interactions may vary by the type of biomaterial and the type of cell and microenvironment. These interactions could be even more complicated when we are dealing with submicron biomaterials in the nano range. I believe that there are many opportunities when playing with nanostructures and nanoparticles in living systems which are still limited

only by our knowledge about the interaction mechanisms. It is now the time to strengthen the interdisciplinary collaborations between engineers, basic, and applied scientists in different fields. This extensive set of chapters in this book depicts work by a variety of world-class researchers on attempts to exploit the specific interactions of biomaterials in the body. The book starts with the basic and fundamental knowledge about biocompatibility. Then, it explains the cellular responses to different classes of biomaterials. In the last part of the book, it deals with the tissue responses to biomaterials. Of course, problems and limitations exist but the breadth of the collaborations in this book indicate that the best is yet to come.

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S E C T I O N I

An introduction to
biocompatibility

Principles of biocompatibility

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1.1 Introduction

There is a growing interest in the field of biomaterials science and engineering due to its vital effects on human health [1]. There are many factors responsible for the efficacy of biomaterials in contact with living tissues and organs [2]. The biomaterials should be able to demonstrate the ability of implantation in the human body without producing an unacceptable degree of harmful effect on the tissue. This is known by the general term “biocompatibility” which is extensively used by biomaterials scientists. However, there is still some hidden aspects about the mechanisms of biocompatibility [3]. The examinations on the first generation of implantable biomaterials indicated that the most efficient biomaterials are those with the minimum chemical reaction in biological environments [4].

Among metallic biomaterials, the plain carbon and vanadium steels were first replaced with stainless steels and further with titanium, platinum, and magnesium alloys, due to their superior advantages in terms of biodegradability and biocompatibility [5]. For polymeric biomaterials, nylons and polyesters were replaced by polytetrafluoroethylene, poly(methyl methacrylate), polyethylene, and silicone, since they are less degradable and toxic. For ceramic-based biomaterials, a range of active glass-ceramics and bioactive glasses have been introduced. This class of biomaterials has attracted great attention due to their ability to incorporate therapeutic ions for the enhancement of biological reactions in the body [6]. In conclusion, biomaterials are usually selected and categorized on the basis that they should not be toxic, immunogenic, thrombogenic, carcinogenic, irritant, and so on, directing us to the definition of biocompatibility [7]. There are a range of material characteristics that greatly influence the host response and further affect the biocompatibility of biomaterials (see Table 1.1) [8]. These characteristics can be divided into two categories related to the bulk and the surface.

Based on the type of biomaterial implanted in the body, a number of reactions can happen over time (see Table 1.2) [8]. In most cases, a sequence of events are involved within

TABLE 1.1 A number of important biomaterial characteristics that can potentially influence the host response.

Bulk material composition
Micro- and nanostructure, morphology, porosity
Crystallinity and crystallography
Water content, hydrophobic–hydrophilic balance
Corrosion parameters and ion release profile
Degradation profile and degradation by-products
Wear debris release profile
Mechanical properties (e.g., stiffness and elastic constants)
SSA
Textural characteristics
Surface chemical composition
Surface molecular mobility
Surface topography
Surface energy
Surface electrical characteristics

SSA, Specific surface area.
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the interface of the biomaterials and tissues. The overall biocompatibility of biomaterials is related to these interactions. The details of these sequential events have been previously explained in the literature [9].

The definition of “biomaterial” has been officially introduced about half a century ago as “a nonviable material used in a medical device, intended to interact with biological systems” [10]. More specifically in 1986, a consensus conference on “definitions in biomaterials: proceedings of a consensus conference of the European Society for Biomaterials” was held in Chester, United Kingdom [11]. At that moment, a wide range of biomaterials have been used in medical devices as inert materials. However, by that time, the ability of biomaterials has been greatly changed to interact with living systems in different ways [12].

The field has been further growing because of the need for emerging applications such as applications in advanced delivery systems, imaging techniques, and regenerative medicine. As a result of this transition shift the field needed to redefine the terms and definitions in a more precise way. In a recent conference in Chengdu, China, 2018, a series of biomaterials experts got together to redefine the most important principles of biomaterials and biocompatibility [13]. According to this expert panel, the term “biomaterial” has been defined as “a material designed to take a form that can direct, through interactions with living systems, the course of any therapeutic or diagnostic procedure”.

TABLE 1.2 The most important host characteristics responses to the implanted biomaterials.

Protein adsorption and desorption
Neutrophil activation
Macrophage activation, foreign body giant cell production, granulation tissue formation
Fibroblast behavior and fibrosis
Microvascular changes
Tissue/organ specific cell responses (e.g., osteoclasts and osteoblasts, endothelial proliferation)
Activation of clotting cascade
Platelet adhesion, activation, aggregation
Complement activation
Antibody production and immune cell responses
Acute hypersensitivity/anaphylaxis
Delayed hypersensitivity
Mutagenic responses, genotoxicity
Reproductive toxicity
Tumor formation

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Among different basic characteristics defined for biomaterials, biocompatibility is of great importance. Biocompatibility can be defined as the ability of a biomaterial with an appropriate host response in a specific application [14–16]. According to this explanation, a biomaterial can interact with biological systems with minimal risk of toxicity and rejection by the immune system.

In the context of host response to biomaterials, carcinogenicity is another important characteristic of an implanted biomaterial, defined as the ability to initiate and/or stimulate the increase of cancerous cells [17].

When a biomaterial is implanted in the body, a series of interactions happen in biological fluids. In most cases, a foreign body capsule is formed on the surface of the biomaterial. This protein layer can greatly change the characteristics of the biomaterial which can further act as a structural and biological barrier between the biomaterial and the tissue [18]. When a biomaterial interacts with blood, a series of even more complex events can happen, where the term hemocompatibility is defined as the compatibility of biomaterials with circulating blood. This interaction should be sustainable without any adverse reactions [19–21]. According to expert opinions, hemocompatibility can be better explained as the ability of a blood-contacting biomaterial to avoid the formation of a thrombus by minimal activation of platelets and of blood coagulation, minimizing activation of the complement system and minimizing hemolysis [13]. In some cases, the implanted biomaterial may also induce some levels of thrombogenicity which is defined as the ability of the biomaterial to stimulate and/or promote the formation of a thrombus in contact with blood [22].

TABLE 1.3 The International Standards Organization (ISO) series of guidance documents for biomaterials and biocompatibility.

Number	ISO number	Title
1	ISO 10993-1:2018	Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process
2	ISO 10993-2:2006	Biological evaluation of medical devices—Part 2: Animal welfare requirements
3	ISO 10993-3:2014	Biological evaluation of medical devices—Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity
4	ISO 10993-4:2017	Biological evaluation of medical devices—Part 4: Selection of tests for interactions with blood
5	ISO 10993-5:2009	Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity
6	ISO 10993-6:2016	Biological evaluation of medical devices—Part 6: Tests for local effects after implantation
7	ISO 10993-7:2008	Biological evaluation of medical devices—Part 7: Ethylene oxide sterilization residuals
8	ISO 10993-9:2009	Biological evaluation of medical devices—Part 9: Framework for identification and quantification of potential degradation products
9	ISO 10993-10:2010	Biological evaluation of medical devices—Part 10: Tests for irritation and skin sensitization
10	ISO 10993-11:2017	Biological evaluation of medical devices—Part 11: Tests for systemic toxicity
11	ISO 10993-12:2012	Biological evaluation of medical devices—Part 12: Sample preparation and reference materials
12	ISO 10993-13:2010	Biological evaluation of medical devices—Part 13: Identification and quantification of degradation products from polymeric medical devices
13	ISO 10993-14:2001	Biological evaluation of medical devices—Part 14: Identification and quantification of degradation products from ceramics
14	ISO 10993-15:2000	Biological evaluation of medical devices—Part 15: Identification and quantification of degradation products from metals and alloys
15	ISO 10993-16:2017	Biological evaluation of medical devices—Part 16: Toxicokinetic study design for degradation products and leachables
16	ISO 10993-17:2002	Biological evaluation of medical devices—Part 17: Establishment of allowable limits for leachable substances
17	ISO 10993-18:2005	Biological evaluation of medical devices—Part 18: Chemical characterization of materials
18	ISO/TS 10993-19:2006	Biological evaluation of medical devices—Part 19: Physicochemical, morphological, and topographical characterization of materials
19	ISO/TS 10993-20:2006	Biological evaluation of medical devices—Part 20: Principles and methods for immunotoxicology testing of medical devices
20	ISO/TR 10993-22:2017	Biological evaluation of medical devices—Part 22: Guidance on nanomaterials

(Continued)

TABLE 1.3 (Continued)

Number	ISO number	Title
21	ISO/CD 10993-23	Biological evaluation of medical devices—Part 23: Determination of skin irritation of medical device extracts using reconstructed human Epidermis
22	ISO/TR 10993-33:2015	Biological evaluation of medical devices—Part 33: Guidance on tests to evaluate genotoxicity—Supplement to ISO 10993-3
23	ISO/TR 15499:2016	Biological evaluation of medical devices—Guidance on the conduct of biological evaluation within a risk management process
24	ISO/DTS 21726	Biological evaluation of medical devices—Application of the threshold of toxicological concern for assessing biocompatibility of extractable substances
25	ISO 14155:2011	Clinical investigation of medical devices for human subjects—Good clinical practice
26	ISO 22442-1:2015	Medical devices utilizing animal tissues and their derivatives—Part 1: Application of risk management
27	ISO 22442-2:2015	Medical devices utilizing animal tissues and their derivatives—Part 2: Controls on sourcing, collection and handling
28	ISO 22442-3:2007	Medical devices utilizing animal tissues and their derivatives—Part 3: Validation of the elimination and/or inactivation of viruses and transmissible spongiform encephalopathy (TSE) agents
29	ISO/TR 22442-4:2010	Medical devices utilizing animal tissues and their derivatives—Part 4: Principles for elimination and/or inactivation of transmissible spongiform encephalopathy (TSE) agents and validation assays for those processes
30	ISO 11737-1:2018	Sterilization of health care products—Microbiological methods—Part 1: Determination of a population of microorganisms on products
31	ISO 11737-2:2009	Sterilization of medical devices—Microbiological methods—Part 2: Tests of sterility performed in the definition, validation and maintenance of a sterilization process
32	ISO 11135:2014	Sterilization of health-care products—Ethylene oxide—Requirements for the development, validation and routine control of a sterilization process for medical devices
33	ISO 7405:2008	Dentistry—Evaluation of biocompatibility of medical devices used in dentistry
34	ISO/TR 37137:2014	Cardiovascular biological evaluation of medical devices—Guidance for absorbable implants
35	ISO 22794:2007	Dentistry—Implantable materials for bone filling and augmentation in oral and maxillofacial surgery—Contents of a technical file
36	ISO 11979-5:2006	Ophthalmic implants—Intraocular lenses—Part 5: Biocompatibility
37	ISO 19227:2018	Implants for surgery—Cleanliness of orthopedic implants—General requirements
38	ISO 5910:2018	Cardiovascular implants and extracorporeal systems—Cardiac valve repair devices

(Continued)

TABLE 1.3 (Continued)

Number	ISO number	Title
39	ISO 15675:2016	Cardiovascular implants and artificial organs—Cardiopulmonary bypass systems—Arterial blood line filters
40	ISO/TS 22911:2016	Dentistry—Preclinical evaluation of dental implant systems—Animal test methods
41	ISO 14630:2012	Nonactive surgical implants—General requirements
42	ISO 17327-1:2018	Nonactive surgical implants—Implant coating—Part 1: General requirements
43	ISO 13175-3:2012	Implants for surgery—Calcium phosphates—Part 3: Hydroxyapatite and beta-tricalcium phosphate bone substitutes
44	ISO/TR 37137:2014	Cardiovascular biological evaluation of medical devices—Guidance for absorbable implants
45	ISO 3826-1:2013	Plastics collapsible containers for human blood and blood components—Part 1: Conventional containers
46	ISO 3826-3	Plastics collapsible containers for human blood and blood components—Part 3: Blood bag systems with integrated features

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There have been always many attempts to systematically assess the biocompatibility of biomaterials. In this regard, there are a range of proposed standard methods for investigating the biocompatibility of biomaterials by International Standards Organization (ISO). [Table 1.3](#) summarizes some of the most important standard methods for the investigation of biomaterials and biocompatibility.

1.2 Conclusion

The understanding toward the exact mechanisms responsible for the cellular interactions at the interface of biomaterials and tissues is not still quite clear. This is very important regarding when a biomaterial should stay for a long-time in the body. More than five decades of research experience in this field indicated that the most important requirement for the biocompatibility of biomaterials is that the biomaterials should not be relatively harmful for the target tissues. By the development of biomaterials for specific applications in tissue engineering, regenerative medicine, niche engineering, immunoengineering, targeted delivery, and critical biological applications, the need for specific interactions at the interface of biomaterials and tissues becomes more essential. Although the knowledge behind the actual mechanisms of biocompatibility on biomaterials surfaces is still in its infancy, this area of research is so attractive that it has fascinated scientists. Undoubtedly, this topic needs further investigations and a lot of critical questions have yet to be answered.

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Bacterial cell–biomaterials interactions

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2.1 Introduction

The use of biomedical implants, such as intravascular catheters, urinary catheters, stents, valves, ventricular assist devices, and endoprosthetic joints, has been an important part of modern healthcare system. Use of implants has revolutionized medicine and increased the quality of life and in some instances, even on the patient survival rates. However, it can also be associated with a variety of complications. One of the most frequent and severe complications associated with the use of implants is biomaterial-associated infection [1,2]. For example, device-associated infections account for 25.6% of all healthcare-associated infections (HAIs) in the United States [3], and the overall direct cost of HAIs to hospitals ranges from \$28 billion to \$45 billion annually [4].

Biomaterial-associated infection is due to the pathogen bacterial adhesion and biofilm formation on medical device surfaces. Bacterial cells adhere to the surface and produce extracellular polymeric substances (EPS), proliferate, and colonize to form biofilms on biomaterial surfaces, resulting in microbial infection. The biomaterial-associated infections are extremely difficult to treat using antibiotics alone. The minimal concentration of antibiotics for eradication of mature biofilm can be up to 100–1000 times higher than for the planktonic bacteria [5]. This is not only because the slime matrix they produce can be physical barriers and protect bacteria from antimicrobial agents [6,7], but also the diverse physiological states of biofilm organisms such as the creation of starved, stationary phase dormant zone in biofilms [8], and the existence of persister cells [9] are the significant factors in the resistance of biofilms to antimicrobials. In addition, biofilm formation may cause

the adverse effect on blood response. In the case of blood-contacting devices, a biofilm may continuously release bacteria into the bloodstream and interact with platelets resulting in platelet activation [10] and thrombus formation [11]. All lead to severe clinical complications often with lethal outcome. Further, a common problem with the long-term treatment with potent antibiotics is the development and continuously expanding population of bacteria resistant to common antibiotics [12–16]. As a result, surgical removal and replacement of the implanted devices is often the only treatment, which naturally cause significant morbidity and mortality, and increase the medical cost [17].

Microbial biofilms are complex consortia of adherent microorganisms embedded in a self-produced polymer matrix of EPS that is composed of polysaccharides, proteins, and extracellular DNA [18]. Development of biofilms is generally proposed as a four-stage model beginning with the adherence of planktonic bacterial cells to surfaces, followed by accumulation, maturation, and dispersal phases (Fig. 2.1) [19–22]. Biofilm formation on surfaces is multifactorial, influenced by the particular strain, the growth environment, and the material surface characteristics. It has been evidenced that capability to form a biofilm, biofilm slime components and structure, and the regulatory biofilm network can be substantially different

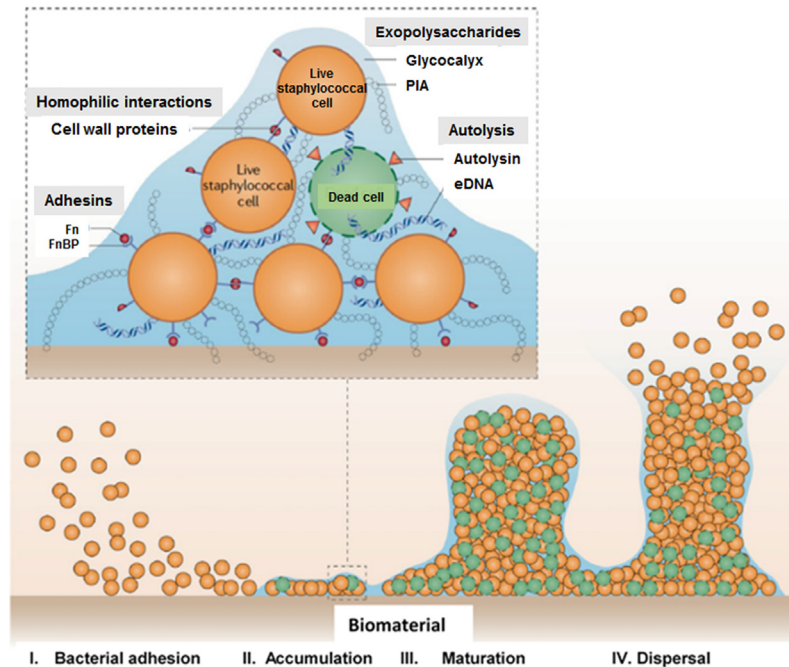


FIGURE 2.1 The schematic representation of Staphylococcal bacterial adhesion and biofilm formation on biomaterial surface. The planktonic cells interact and anchor on biomaterial surface. Intercellular interactions mediated by adhesins and cell wall proteins lead bacteria to cluster together and form microcolonies. The production of EPS matrix composed of proteins, glycoproteins, glycolipids, polysaccharides, and eDNA facilitates the formation of the biofilms. eDNA, Extracellular DNA; EPS, extracellular polymeric substances. Source: *Reproduced and modified from Arciola CR, Campoccia D, Montanaro L. Implant infections: adhesion, biofilm formation and immune evasion. Nat Rev Microbiol. 2018;16(7):397–409 with permission from Nature Publishing Group.*

between clinical isolates of the same species [23]. This biological variability has to be addressed in the understanding of biofilm formation as well as the development of antibiofilm strategies [24]. The most common microorganisms diagnosed in the nosocomial infection include *Staphylococci*, *Enterococci*, *Pseudomonas*, and *Candida* species [13,25]. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli* are mostly isolated strains from IV catheters in catheter-related bloodstream infections [26–28]. Cluster-forming Gram-positive *S. aureus* has received significant attention and has been intensively studied in infection [29–32] because it is much more virulent and aggressive than others. It synthesizes an array of toxins and other virulence factors, causing a range of acute and pyogenic infections. However, *S. epidermidis* ranks first among the causative agents of nosocomial infections and represents the most common source of infections on indwelling medical devices such as prosthetic heart valves and joint prostheses [33,34]. The main defined virulence factor associated with *S. epidermidis* is its ability to colonize on biomaterials and form biofilms which are recalcitrant to the deleterious action of antibiotics and impede the host immune response [35].

2.2 Theoretical theories of bacterial adhesion to biomaterial surfaces

Bacterial adhesion to the device surface is the first and critical step in the pathogenesis of implant-related infections, involving complex interactions between the pathogen and the biomaterials. The planktonic bacteria in bulk fluid are freely suspended before attaching to surface, and are transported to a substratum surface through flow of the fluid or mass transport processes such as convection, diffusion, or sedimentation. Upon contacting surface, bacterial attachment to implant surface can be divided into two phases, that is, the initial instantaneous and unspecific reversible physical phase; and a specific irreversible molecular and cellular attachment phase [36]. Initial attachment is rapid for a short period of time on the order of ~ 1 minute, and includes hydrodynamic and physicochemical interactions [37,38]. Long range (Lifshitz–van der Waals and electrostatic forces) and short range (Lewis acid–base) forces are generally involved in the initial attachment process. The second phase of bacterial attachment is irreversible and can occur on a time scale of several hours. Molecular and cellular molecular reactions between cell surface and substratum surface become predominant in this phase. A firmer adhesion of bacteria to a surface is formed by the bridging function of bacterial surface polymeric structures including capsules, fimbriae or pili, and slime [39]. Irreversible attachment is facilitated by the production of EPS. The polysaccharide adhesins, clumping factors, and proteins are often involved in this process [40,41].

Initial bacterial attachment is determined by the physical and chemical interactions, depending upon the complex interplay of the physical and chemical properties of the bacterial and substratum surfaces in aqueous. Various theoretical approaches have been made to explain the mechanism of bacterial adhesion. The main theories include thermodynamic approach, DLVO (named after Derjaguin, Landau, Verwey, Overbeek) theory and extended DLVO (XDLVO) theory.

The thermodynamic theory is based on the measurements of interfacial energy between two interfaces (liquid–solid, bacteria–liquid, and bacteria–solid) to calculate the Gibbs

free energy of the system, thereby explaining bacterial adhesion to surface [42]. Adhesion is favored if the Gibbs free energy is negative, otherwise, coadhesion will be predicated. This approach has been proven accurate in correlation to bacterial adhesion only in a few cases [43] but is generally regarded too simplistic and inaccurate.

The behavior of many bacteria during interactions and adhesion to abiotic surfaces can be regarded as colloids because the bacterial cell surface properties (e.g., size being about 0.5–2 μm and shape) typically resemble those of colloids [44]. The theoretical models of colloidal adhesion such as DLVO theory or XDLVO theory [45,46] are often used to describe the bacterial adhesion process. In the classical DLVO theory, microbial cells are assumed to behave as inert particles. The Lifshitz–van der Waals and electrostatic interaction forces between two particles immersed in a medium are considered for the calculation of total energy of the system, and each system will develop towards the energy minimum. Both forces can be attractive or repulsive, depending on the properties of particles. Further, the sign of such forces can also change with the separation distance. Such approach has been employed to predicate the colloidal stability and successfully predicted the biofilm capability of microorganisms on different substrates [46]. However, many other interfacial phenomena also play an important role in bacterial adhesion, for example, hydration and hydrophobicity, which are not included in DLVO theory. In order to take into consideration other phenomena involved in colloidal interactions, the XDLVO theory was developed. The extend DLVO theory considered not only the long range Lifshitz–van der Waals and electrostatic interactions but also the short range Lewis acid–base interactions. The sum of these three interactions between two surfaces is used to calculate the total free energy of the system. Since the Lewis acid–base component indicates the potential formation of covalent bonds between two surfaces which play the most important role in bacterial adhesion to surface, XDLVO theory provides a better predication than DLVO in assessing bacterial adhesion [45]. However, it is not always accurate to predict the behavior of viable bacteria using either DLVO or XDLVO theories. This is mostly due to the complex properties of bacterial cell surfaces, which are often covered with exopolymers leading to interactions between polymers and surface. Other factors such as surface heterogeneity, fluid flow, and surface roughness also contribute to bacterial adhesion that DLVO and XDLVO theories fail to predicate [47].

2.3 Factors influencing bacterial adhesion to biomaterial surfaces

Bacterial adhesion to biomaterial surfaces involves the interactions among three phases (strain, substrate, and liquid environment) and is influenced by many factors from these phases (Fig. 2.2) [48]. They include the bacteria factors (Gram-positive or -negative, surface energy and charge, outer membrane molecular details), the biomaterial surface factors (chemical composition, roughness, topography, surface energy, and charge, etc.), and environmental factors (serum proteins, flow conditions, temperature, bacterial concentration, time of exposure, antibiotics). The properties of each factor can be interconnected, and the physical and/or chemical changes in one factor can have an impact on the others. The study of bacterial adhesion to surface should consider and integrate these factors in

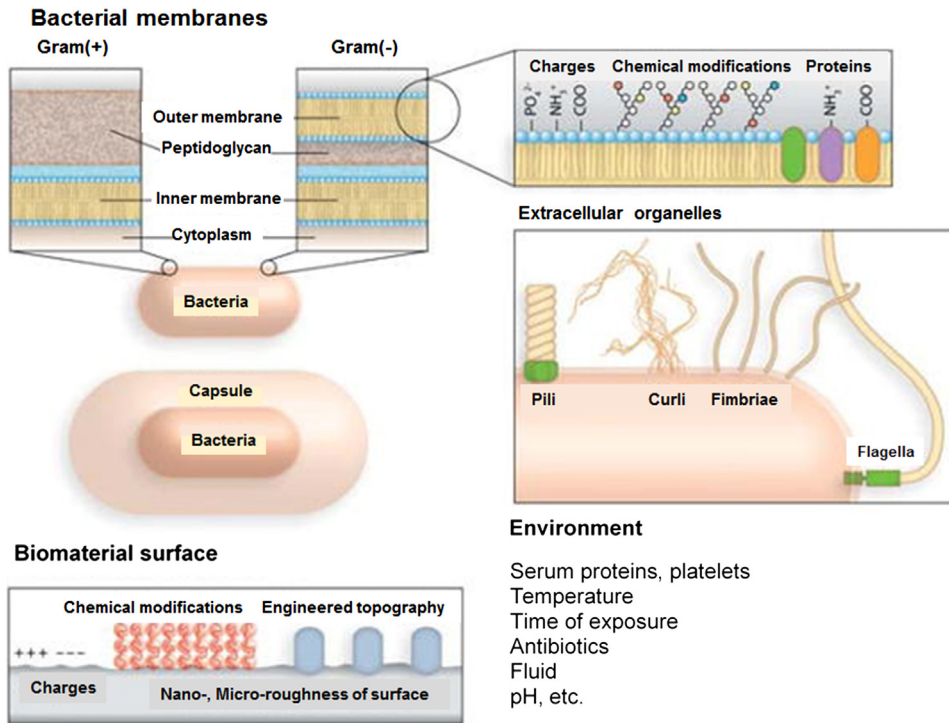


FIGURE 2.2 Factors that influence the interactions between bacterial cells and biomaterial surfaces. The cell wall of Gram-positive bacteria consists of an inner lipid membrane surrounded by a layer of peptidoglycan and the Gram-negative bacteria cell wall consists of an inner lipid membrane surrounded by a layer of peptidoglycan, which is surrounded by an outer lipid membrane. Outer membrane proteins and lipopolysaccharide provide surface charge. Some bacteria have extracellular organelles including pili, curli fiber, and flagella that facilitate attachment and motility. Biomaterial surface properties include charge, hydrophobicity, topography, and the chemical compositions. Environmental parameters such as serum protein adsorption, platelets, temperature, fluid condition, and antibiotics affect bacterial interactions with surfaces. Source: Reproduced and modified from reference Renner Lars D, Weibel Douglas B. *Physicochemical regulation of biofilm formation*. MRS Bull 2011;36(5):347–55 with permission from Cambridge University Press.

complexity. Some reviews on the interactions of bacteria and surface can be found elsewhere [39,49,50] and the main factors influencing bacteria-biomaterial interactions are discussed below, including biomaterial surface properties, plasma proteins, platelets, and fluid flow.

2.3.1 Biomaterial surface properties

A broad range of biomaterials including naturally derived, synthetic, and the semi-synthetic or hybrid materials, have been widely applied in medical devices and suffered with microbial infection [51]. The huge variability in material surface compositions and surface properties determines the complexity of bacterial behaviors on surfaces. The biomaterial surface properties, such as surface chemistry, roughness, surface energy, and

surface charge, are known to be the major factors influencing initial bacterial adhesion and biofilm formation on implant surfaces [52–54]. Understanding how surface properties influence bacterial adhesion is crucial to identify the biomaterials with the potential for microbial infections, and also to develop novel biomaterials or coatings with the antifouling characteristics.

Surface chemical composition determines physicochemical properties of substratum surface and moderates the bacterial adhesion. As discussed before, the adhesive forces between abiotic substrate and bacterium arise through van der Waals and electrostatic double-layer interactions as well as the acid–base interactions, and the bacterial adhesion can be predicated by theoretical models. To gain deeper insight into the influence of surface chemistry on bacterial adhesion, it is important to minimize the substrate roughness effect, and the experimental surfaces should be molecularly smooth, generally with a root-mean-square roughness less than 2 nm [55]. To reach this experimental condition, self-assembled monolayers (SAMs) of thiol thin layer on gold substrate or alkyl silane layers on glass substrate are often used to obtain surfaces exposing different functional groups. Tegoulia and Cooper [56] utilized thiol surfaces to study the adhesion of *S. aureus* and found that adhesion was the lowest on the ethylene oxide-bearing surfaces, followed by the hydroxyl surfaces, while adhesion on the carboxylic- and methyl-terminated SAMs was much higher. Oh et al. [55] investigated the effects of substrate hydrophobicity and zeta potential on the dynamics and kinetics of the initial stages of bacterial pathogens *S. aureus* and *E. coli* O157:H7 on the gold surfaces coated with thin thiol layers. These model thiol surfaces have precisely controlled surface chemistry and thickness with varying hydrophobicity and surface potential. The researchers found that bacterial adhesion was greatest on hydrophilic substrates with positive surface charge characteristics, followed by hydrophobic substrates with negative surface charge characteristics, and the smallest on hydrophilic substrates with negative surface charge characteristics. In the other study of *S. epidermidis* adhesion on the alkyl silaned glass surfaces, the CH₃ terminated surface with water contact angle of $93.0 \pm 3.2^\circ$ produced the highest adhesion, followed by the positively charged NH₂ functionalized surface, the noncharged NH₂ groups (water contact angle $49.1 \pm 3.3^\circ$), the COOH (water contact angle $32.8 \pm 1.9^\circ$), and minimal on the OH-terminated glass [57]. Results suggested that the increase in the material surface's free energy reduced the adhesion of a hydrophilic bacterial strain, and this is in accordance with the predictions of the thermodynamic theory [58].

On the polymeric biomaterial surfaces, bacteria adhesion is also largely influenced by surface chemistry. The Anderson group studied the adhesion of *S. epidermidis* on surface-modified polyethylene terephthalate (PET) biomaterials with varying surface charge and hydrophobicity. The results showed that hydrophilic surface had significantly less nonspecific adhesion of bacteria than that in the control (PET) and other surfaces when cultured in phosphate-buffered saline (PBS), however, charged surfaces, both anionic and cationic, had increased adhesion and aggregation of bacteria in comparison with the control (PET) in the presence of serum proteins [59]. The same group also studied the adhesion of *S. epidermidis* on polyurethanes (Elasthane 80A, hydrophobic) biomaterials which were modified with polyethylene oxide (Elasthane 80A-6PEO, hydrophilic) and fluorocarbon (Elasthane 80A-6F, hydrophobic) [60]. Modification of polyurethane with polyethylene

oxide significantly inhibited *S. epidermidis* biofilm formation over 48 hours in vitro. Yuan et al. found the polymeric biomaterial with moderate hydrophobicity (water contact angle of about 90 degrees) produced the highest level of *E. coli* adhesion while the extremely hydrophobic or the extremely hydrophilic surface reduced adhesion [61]. This finding appears to be conflicted with the general bacterial adhesion trends with respect to the surface chemistry, that is, the extent of bacterial adhesion generally increases with increasing hydrophobicity and decreasing surface energy of abiotic surfaces for hydrophilic bacteria [62,63]. The reasons for the conflict might be multiple and complicated, however, the substrate surface roughness and surface topography may be important to alter the bacterial adhesion behavior in interaction with surfaces [55].

Surface roughness is a two-dimensional parameter of a material surface and is generally characterized as the value of arithmetical mean deviation of the height profile (R_a) or root mean square (RMS) roughness (R_q). A large surface area with rough topography can promote bacterial adhesion and harbor more biofilms loads because a rough surface produces a greater surface area and provides more favorable sites for colonization adhesion [64–66]. However, the effect of surface roughness on adhesion is apparently related to the degree of roughness, topographic features of surface, and material compositions. Yoda et al. [67] investigated *S. epidermidis* adhesion onto five types of metallic biomaterial surfaces, including oxidized zirconium-niobium alloy (Oxinium), cobalt–chromium–molybdenum alloy (Co–Cr–Mo), titanium alloy (Ti–6Al–4V), commercially pure titanium (Cp–Ti) and stainless steel (SUS316L), with different levels of roughness below 30 nm R_a , and found that the minimum level of roughness affecting initial bacterial adherence activity differs according to the type of biomaterial used. A surface roughness of below 30 nm R_a in Oxinium, Ti–6Al–4V, and SUS316L can promote bacterial adhesion, while the coarse Co–Cr–Mo materials exhibited significantly lower values. Bollen et al. provided a “threshold R_a ” that a reduction in surface roughness on titanium surface below $R_a = 0.2 \mu\text{m}$ had no effect on microbial adhesion or colonization [68,69]. Tang et al. reported that surface roughness significantly affects the adhesion of *S. epidermidis* on silicon surfaces only when the root-mean-square roughness (R_q) was larger than 200 nm [70]. The studies of influence of physical structuring of surface nanostructures on bacterial adhesion further revealed the role of surface roughness in bacterial adhesion. Whitehead et al. [71] measured the bacterial adhesion on Si wafers coated with titanium having featured surface pit sizes of 0.2, 0.5, 1, 2 μm in diameter and different depths, resulting in R_a values ranging from 45 to 220 nm. Results confirmed that adhesion generally increased with surface roughness, however, surfaces with 500 nm diameter pits showed the lowest number of bacterial adhesion due to the reduction of the contact area for bacterial interaction and the change of surface energy by roughness. It should be pointed out that the most commonly reported surface roughness parameters (R_a and R_q) are only the measures of the typical height variation of the surface and they offer no insights into the spatial distribution or shape of the surface features. To more accurately characterize surface roughness at the nanoscale, a standard set of topographical parameters such as RMS surface roughness, summit density, developed area ratio, the ten-point average roughness, skewness, texture aspect ratio, and bearing ratio may be adopted to predicate the bacterial cell adhesion behavior, which will have a profound impact on the design and fabrication of biomedical implants [72].

2.3.2 Plasma proteins

When a foreign material is implanted in the body, plasma proteins rapidly interact with the surface and form a protein layer. The nature of adsorbed proteins on biomaterial surfaces affects the initial bacterial adhesion and alters the process of biofilm formation. This adsorbed protein layer may minimize the effect of biomaterial surface properties on bacterial adhesion [56,73], instead, interactions between the bacteria and the proteins become more important in bacterial adhesion. The serum proteins generally suppress initial bacterial adhesion due to the lack of a specific interaction between albumin and bacteria [60,74] while adhesive plasma proteins such as fibrinogen (Fg) and fibronectin (Fn) promote bacterial adhesion [56,75,76]. This increase in bacterial adhesion is believed to be due to specific ligand/receptor events between plasma proteins and bacterial cell surface proteins known as the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) [40,77–79] or termed bacterial adhesins [80]. A variety of cell surface proteins have been identified on Staphylococcal strains that have the ability to bind many plasma proteins including Fg [56], Fn [81], vitronectin [82,83], and von Willebrand factor (vWF) [84].

Fg is the third most abundant protein plasma in blood and plays a prominent role in development of surface-induced thrombosis [85,86] but also promotes the adherence of bacteria such as *S. aureus* [87]. Tegoulia and Cooper investigated the adhesion of *S. aureus* on Fg preadsorbed SAMs coated surfaces and found that preadsorption of Fg minimized the effect of the surface chemistry on bacterial adhesion, but increased the adherence of *S. aureus* on all SAMs surfaces [56]. The enhancement of *S. aureus* adhesion by Fg is due to the formation of receptor–ligand bonds between protein and cell surfaces. MSCRAMM clumping factors A (ClfA) and B (ClfB) on *S. aureus* cell surfaces have been identified to bind to Fg [40,41]. The recent study shows that ClfA is a force-sensitive molecular switch that potentiates staphylococcal adhesion under mechanical stress [88]. The dramatically enhanced force between ClfA and immobilized Fg by mechanical tension may explain that ClfA promotes bacterial attachment under high physiological shear stress. Fg is also reported to enhance *S. epidermidis* adhesion. A Fg-binding adhesin gene was found on *S. epidermidis* cell surfaces from central venous catheters-associated and orthopedic implant-associated infections, demonstrating that Fg increased the adhesion of *S. epidermidis* to biomaterial surfaces [89]. Similarly, an Fg-binding bacterial adhesin called SdrG was identified from *S. epidermidis* cell surface and promoted bacterial adhesion [90]. The cell surface protein SdrG mediates adhesion of *S. epidermidis* to Fg through a specific “dock, lock, and latch” structural model, which results in greatly stabilized protein–ligand complexes [91]. This mechanism represents a general mode of ligand binding for structurally related cell wall-anchored proteins of Gram-positive bacteria. Vanzielegheem et al. [92] further investigated the surface density of SdrG on *S. epidermidis* cell surfaces using single molecule force spectroscopy technique and found that *S. epidermidis* affinity for Fg-coated surfaces correlates well with the increased amounts of SdrG adhesin on the cell surface. These results provided direct evidence that abundance of SdrG on the cell surface dramatically improves their ability to bind to Fg-coated implanted medical devices resulting in the increase in adhesion.

Fn is another major plasma protein influencing bacterial adhesion on implanted biomaterials. Fn is a large multidomain glycoprotein with multiple adhesive properties and

functions as a key link between cells and their extracellular matrices. It can bind a variety of extracellular molecules such as fibrin, heparin, and collagen, and plays a key role in cell adhesion and proliferation [93,94]. However, the effect of Fn on the adherence of bacteria is highly variable. On one hand, numerous studies have found that Fn facilitates bacterial adhesion to biomaterials including *S. aureus* and *S. epidermidis* [75,95–97]. Similar to the mechanism of enhancement of bacterial adhesion by Fg, fibronectin-binding proteins have been identified in both Gram-positive and -negative bacteria [98,99], including *S. aureus* [100] and *S. epidermidis* [101], and contribute to bacterial adhesion in the presence of Fn. On the other hand, several studies showed that Fn had no effect or even inhibited the adhesion of *S. epidermidis* to protein-coated surfaces [102–104]. Inhibition of adhesion is likely because the production and interaction of an exopolysaccharide by *S. epidermidis* that influences interactions with protein-coated surfaces [102] or the adhesion of *S. epidermidis* to Fn-coated surface is not a specific adhesion [104]. The conflicting results indicated the most basic mechanisms of binding between *S. epidermidis* and Fn-coated surface are unknown yet, and more work should be done to examine the role of Fn in adhesion of bacteria to surfaces, especially in sight of interactions of protein-cell at molecular level on substrata with different physicochemical properties.

Conformational structure and orientation of plasma proteins immobilized on surfaces such as Fg and Fn influence bacterial adhesion. Fg is a glycoprotein composed of two trimers with each trimer composed of three differently intertwined polypeptide chains ($A\alpha$ or α chain, $B\beta$ or β chain, and γ chain). The Fg-binding MSCRAMM ClfA from *S. aureus* interacts with the C-terminal region of the Fg γ -chain [105], while ClfB of *S. aureus* was found to bind to a short region within the C terminus of the Fg α -chain [106]. Fn is a dimer of two similar polypeptides linked by disulfide bonds at the carboxyl terminus, and possesses several functional domains that bind to a variety of extracellular molecules such as heparin and collagen [98]. There are two physiologically relevant binding sites to adhere to bacterial cell surface, which are located at the N-terminus and the C-terminus of Fn. *S. epidermidis* was shown to exhibit a different adhesion response when Fn was oriented with the C-terminus versus the N-terminus bound to the surface [107]. Holmes et al. studied the bacterial interactions with intact or fragments of Fn by surface plasmon resonance technique and found *S. aureus* preferentially binds to the Fn N-terminal region whereas *S. epidermidis* binds to the C-terminal domain [108]. Vellido-Rodriguez et al. found that Fn molecules adopt a more extended conformation on hydrophobic than hydrophilic Ti–6Al–4V surfaces and the extended conformation of the proteins facilitates the exposure of specific sites for adhesion, resulting in higher bacterial attachment [109]. The development of immuno-AFM (atomic force microscopy) technique provides the approaches to assess the effect of protein orientation on bacterial interaction at the molecular scale. The orientation of Fn adsorbed on polymeric biomaterial surface can be detected by the monoclonal antibodies (MAb)-coupled AFM probes [110]. Results showed that Fn adsorbed on polyurethane surfaces from pure Fn solutions showed more C-terminus available than N-terminus in the same sample, and corresponded to higher bacterial adhesion, indicating that *S. epidermidis* bacterial cell surface prefers to bind at the domain of C-terminus of Fn. Furthermore, the presence of albumin influences the orientation of Fn and more N-terminus is available, although the amount of Fn adsorption is lower in this case.

2.3.3 Platelets

Pathogenic bacteria can occasionally enter the human circulatory system and lead to the interaction of bacterial pathogens with platelets, resulting in life-threatening diseases such as stroke. Interaction of bacteria and platelets can induce platelet activation, which is a necessary step in thrombus formation. On the other hand, the adhered platelets can also promote bacterial adhesion on biomaterial surface [111]. For example, Wang et al. studied the adhesion of *S. epidermidis* on hydrophobic polystyrene surfaces under well-defined shear conditions approximating human blood circulation by using a rotating disk system and found that contact-activated platelets *S. epidermidis* adhesion to polyethylene is mediated by contact-activated platelets, not adsorbed plasma proteins. The platelets increased adhesion more than on a protein adsorbed surface by at least one order of magnitude [112].

Numerous studies showed that bacterial surface proteins and plasma proteins are involved in platelet-bacteria interactions [10,113–115]. The former includes the clumping factors, Fn-binding proteins of *S. aureus* [116,117], and Fg-binding protein SdrG of *S. epidermidis* [118]. Cox and coworkers reviewed the bacteria-platelet interactions and summarized that interactions can be characterized by the binding of bacteria to platelets either directly through a bacterial surface protein or indirectly by a plasma bridging molecule that links bacteria and platelet surface receptors [119,120]. Platelets are activated by interaction with bacteria via either direct interaction or indirectly through bridging molecules or the secreted bacterial products. Bacteria may be enhanced to adhere to biomaterial surfaces.

Plasma proteins influence interaction of the bacteria-platelet on the biomaterial surface. The interaction of the platelet and bacteria causes the activation of platelets and forms the aggregates of platelet-bacteria which activated platelets are either adhered with bacteria or entrapped in bacteria cluster. The presence of human serum albumin (HSA) generally decreases the amount of bacteria-platelet aggregates on biomaterial surface, while the presence of Fg or Fn largely increases the formation of aggregates. Furthermore, HSA inhibits the activation of the platelet, even the existence of bacteria and nonactivated platelets can be observed on the biomaterial surface. On the contrary, Fg or Fn promotes the activation of platelets and serves as the linker in interaction of bacteria and platelets, leading to aggregates [21].

2.3.4 Fluid flow

Fluid flow produces a hydrodynamic force and affects the bacterial behaviors on the surface at multiple length scale, from single bacteria adhesion to the development of multicellular bacterial communities such as biofilms [121]. Fluid flow not only controls the initial attachment of bacteria to surfaces [122,123], but also may influence quorum sensing regulation [124–126], growth rate modification [127,128], metabolic pathway changes [129], extracellular matrix production [130], enzymatic reaction [131], and possibly modification of bacterial gene expression, physiology, and pathogenesis [132,133], thereby altering the bacterial behavior at material surface and biofilm formation as well as 3D biofilm structures. Usually, the shear stress produced by flow at a solid–liquid interface can easily

overcome the adhesive forces for attaching cells onto a surface, and potentially detach them from substrata, resulting in a decrease in bacterial adhesion. In the early study, Vacheethasane et al. [73] found that four clinically isolated *S. epidermidis* strains showed the decrease in adhesion to polyethylene surfaces in the shear stress regime 0–15 dyn/cm², and low adhesion (<1% adhesive coefficient) was observed at higher shear stresses. Shear stress also affected bacterial adhesion onto polyetherurethane urea biomaterial surfaces and showed a linearly decrease with increasing shear stress in PBS under shear stress (0–17.5 dynes/cm²) [134]. Apparently, a decrease in bacterial adhesion by shear stress is due to the increase in detachment force produced by fluid flow. At the single cell level, Thomen et al. discovered the threshold value of shear stress that determined biofilm settlement, and above a threshold shear stress value of 10 mPa, no direct initiation of the biofilm on the surface could occur, suggesting very weak forces in the order of sub-picoNewton, sufficient to break the single molecular bond attaching a bacterium to a surface and prevent biofilm initiation [135].

Paradoxically, on the other hand, many examples were also reported, which showed increasing shear stress enhances cell adhesion to surfaces. This is partially due to an increase of microbial and mass transports to a substratum surface by fluid flow velocity increases [136]. Saur et al. found that high wall shear stresses increased the quantity of attached bacteria but also altered their spatial distribution on the substratum surface, and the adhered bacterial communities changed gradually with the applied shear [137]. When fluid flow exceeds a critical limit, wall shear rates may become high enough to prevent adhesion or even stimulate detachment [138]. The Reynolds number (*Re*) is an important number for describing the flow conditions and indicating changes from laminar to turbulent. Kim et al. [128] developed the dimensionless biofilm development model with *Re* number in microfluidic channels and found that higher flow rates encouraged growth of biofilms at low *Re* and higher flow rates with high *Re* suppressed growth of biofilms.

The enhancement of bacterial attachment by shear stress is mostly regarded to be attributed from the result of the formation of catch-bonds between cell and surface, while shears stress enhanced the individual cell catch-bonds [139]. Thomas et al. [140] observed that shear stress enhanced the adhesion of *E. coli* to mannose-coated surfaces and they speculated that it was due to the formation of catch-bonds between bacteria type I pili adhesin called FimH and surface-attached mannose and the bond strength was increased by shear stress [141]. Shear stress also enhanced the attachment properties of *P. aeruginosa*. Lecuyer et al. [123] discovered that residence time of adhesion events of *P. aeruginosa* increased approximately linearly as the shear stress increased in the range of 0 ~ 3.5 Pa. However, *P. aeruginosa* employed different mechanism with *E. coli*. The authors used mutant strains defective in surface organelles (type I pili, type IV pili, or the flagellum) or extracellular matrix production, and found that surface chemistry is not responsible for the trend in the shear-enhanced adhesion time although surface features influenced the frequency of adhesion events and the early-stage detachment probability. The findings suggested an alternative mechanism of shear-dependent adhesion whereby multiple adhesive structures participated to increase surface attachment response to shear stress [121]. The shear dependent adhesion by formation of catch-bonds has also been observed on a variety of bacterial strains. Weaver et al. [142] characterized two modes of attachment of *S. epidermidis* to human fibrinogen (Fg)-coated surface and found that single

colonies adhered in high fractions at low shear stresses (~ 1 dyne/cm² or ~ 0.1 Pa) while clusters of bacteria adhered the highest at median wall shear stress (up to 10 dyne/cm² or 1 Pa). Both of these modes of attachment are dependent on Fg and are determined from the specific molecular recognition between the bacteria and human fibrinogen. *S. aureus* [143] and *Staphylococcus lugdunensis* [144] cell surfaces were found to interact with vWF, a high molecular weight protein in blood, to adhere on the surfaces. When surface bound vWF is exposed to the blood flow under high shear stress, the resulting shear forces unfold the protein and make the receptor–ligand bonds available for bacterial cells, resulting in the adherence of strains to the vessel wall and leading to severe infections such as endocarditis.

2.4 Bacterial interaction with antibacterial biomaterial surfaces

Although in most cases antibiotic is the main strategy to treat biofilm infections, the extensive use of antibiotics worldwide has led to an antibiotic resistance crisis where a large number of bacteria have developed resistance against conventional antibiotics [145,146]. Therefore antimicrobial strategies supplemental to antibiotics during the last few decades have focused on the antibacterial surfaces, either the fabrication of new surfaces or the improvement of the performance of existing antibacterial surfaces. Antibacterial surfaces can be broadly classified as antibiofouling surface that hinders bacterial adhesion and biofilm formation, and bactericidal surfaces that kill bacteria either by contact or release of antimicrobial agents [147]. In some cases, antibacterial surfaces may exhibit dual functions that both kill bacteria and resist bacterial adhesion. These antibacterial surfaces significantly reduce the attachment of bacteria and inhibit biofilm formation, thereby largely reducing the use of antibiotics. In the following sessions, we will talk more about the antibiofouling surfaces with surface topography modification since it is a nonpharmaceutical approach to control the colonization of microbes without causing any resistance.

Antibiofouling surfaces may resist or prevent cellular attachment due to the presence of a functional material surface that has the unfavorable surface topography or surface chemistry with respect to the microorganisms [148]. A wide range of surface treatments have been developed to generate a functional surface to reduce microbial contamination in the healthcare setting [149]. Among these material surface topography modification presents a promising approach and is more attractive because the topographic surface applied to biomaterials does not require modification of surface chemistry and mechanical properties of bulk materials, but does significantly reduce bacterial adhesion and biofilm formation. More importantly, surface topographic modification does not require the addition of antibiotics which often cause resistance, it is significant to the current antibiotic resistance crisis [150]. Clues for prevention of biofilm formation by surface topography are inspired from natural antifouling surfaces such as shark skin, mussel surface, insect wings, and lotus leaves [151–155]. The endothelium of a healthy artery is another example of a natural antifouling system [156]. These natural surfaces are generally microtopographically structured and possessing superhydrophobic (water contact angle >150 degrees) and self-cleaning characteristics, creating an ideal antifouling surface. With interest in this area and the related research increased, biomimetic or bioinspired materials and methods have

been developed over the last two decades, which have been extensively reviewed in the literature [157–160].

Structural topography is created by either depositing material on a surface or etching away part of a surface. Using a variety of chemical and mechanical methods a large number of topographic surfaces, either regularly patterned or irregular/random patterns in the microscale or nanoscale, have been fabricated to prevent microbial contamination on surfaces [155]. The well-defined patterned surfaces generally consist of the ordered and periodic geometry features, for example, pits, pillars, grooves, ribs, or ridges, with the size in nano or micrometer scale. For example, materials with rib features mimicking the shark skins at certain length scales termed as “sharklet” have shown increased resistance to marine biofouling [161,162]. These bioinspired antifouling materials generally possess micron size structured surfaces having dimensions ranging from 1 to 300 μm and the specific patterns [163]. The creation of engineered nanoforce gradients on this specific patterned surface was believed to contribute to the low attachment of microorganisms in the marine environment [164]. Similar topography was attempted to be applied onto poly (dimethylsiloxane) (PDMS) elastomer surfaces and it was found that the surface disrupted biofilm formation of *S. aureus*, and early biofilm colonization weren’t observed on topography surfaces until day 21, compared to 7 days biofilm colonization on smooth surfaces (Fig. 2.3) [165]. These results evidenced the possibility of surface topography modification for application in biomedical devices. The further study showed that the Sharklet micro-pattern applied in a central venous catheters-relevant thermoplastic polyurethane significantly reduced bacterial colonization and relevant platelet interactions after simulated vascular exposure [166].

The surface topography features influence bacterial interaction with surfaces. Material surface roughness is known to be one of the key factors in determining the extent of bacterial colonization, which has been discussed in a previous section. The other surface features affecting bacterial adhesion include feature size and shape, the distance between microfeatures and their organization. The engineered surfaces with well-defined patterns, for example, grooves, may orientate and align the cell’s attachment on surface while no orientation of cells was visible on the random nanometer structure surfaces [167]. Furthermore, it’s also reported that the incorporation of a series of parallel microfabricated grooves significantly impeded active biofilm expansion and reduced biofilm formation [168]. Gu et al. [169] investigated the adhesion of *E. coli* on top of 5 μm tall line patterns with varying width and inter-pattern distance, and found that cells prefer to align perpendicularly to the direction of narrow line patterns, but the orientation of cells became more random when line patterns got wider. They found that the cell cluster formation on 5 μm wide line patterns was reduced by 14-fold compared to flat surface and suggested that such unfavorable topography may present a stress to attached cells. In the other study of a 10 μm -tall hexagon-shaped topographic pattern with a side length of 15 μm and interpattern distance of 2 μm , it was found that the topography reduced biofilm formation on the side of protruding patterns and interrupted cell–cell interaction in the grooves and in total of 85% biofilms were reduced compared to the smooth surface [170]. All of these works indicated the importance of surface features in the design of engineered surfaces to inhibit bacterial adhesion on medical implants. Vadillo-Rodríguez group addressed the adhesions of three bacterial strains (*S. epidermidis*, *E. coli*, and *Bacillus subtilis* representing different Gram-stains and shapes) on a

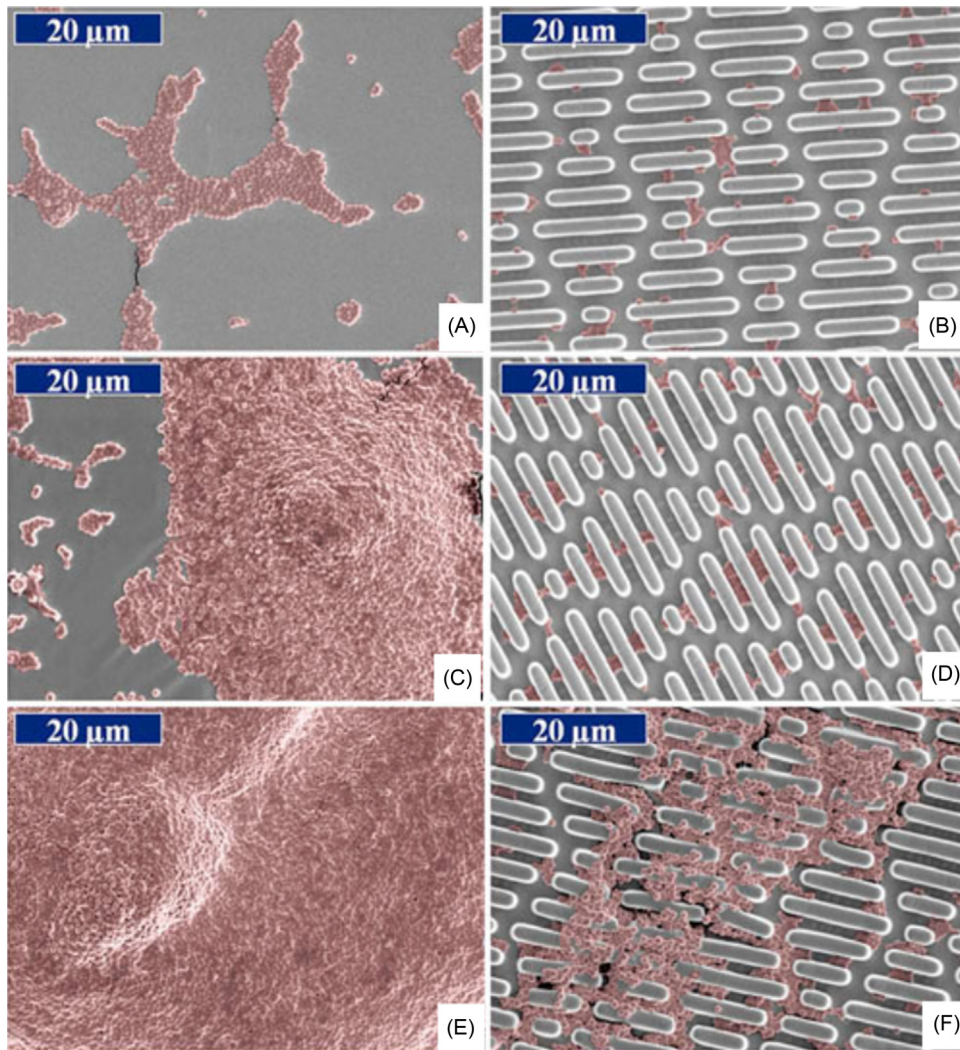


FIGURE 2.3 SEM images of *S. aureus* on smooth (left) and engineered topographically modified (right) PDMS surfaces on day 7 (A, B), day 14 (C, D), and day 21 (E, F). PDMS, Poly(dimethylsiloxane). Source: Reproduced from Chung KK, Schumacher JF, Sampson EM, Burne RA, Antonelli PJ, Brennan AB. Impact of engineered surface microtopography on biofilm formation of *Staphylococcus aureus*. *Biointerphases* 2007;2(2):89–94 with permission AIP Publishing.

range of spatially organized microtopographic surface patterns generated on PDMS [171]. They found that bacterial cells were able to differentiate upper and lower areas in spatially organized microtopographic surface patterns and the selective adhesion depended on the size and shape of the cells relative to the dimensions and height/depth of the topographical features and on surface hydrophobicity/hydrophilicity. Furthermore, they found that all the topographies investigated provoked a significant reduction in bacterial adhesion relative to the smooth control samples regardless of surface hydrophobicity/hydrophilicity. In the

recent study of *Staphylococci* strains adhesion on the PDMS surfaces with spatially organized microtopographic surface patterns with nanometer scale roughness, the same research group found that bacterial cells preferably adhered to the square corners and convex walls of recessed surface features rather than the flat or concave walls of equal protruding features to maximize the cell-surface contact points in the initial adhesion events. These findings suggested that particular geometry of the features employed, for example, the well-defined nanoscale topography such as inner or outer square corners, the curving in, out or null of the vertical wall surfaces, could be used to control the initial location of the adhering cells and the process of biofilm formation [172]. Again, bacterial adhesion and biofilm formation were also found to be statistically significantly reduced on these surface patterns, demonstrating that engineered topographies with nanometer scale roughness are effective at inhibiting bacterial adhesion and colonization.

Titanium and titanium alloys are one of the most widely used implant materials for a range of medical applications, including orthopedic and dental implants, due to their biocompatibility, chemical inertness, and excellent resistance to corrosion. However, their performances can exhibit significant complications both in the short and long terms, and are adversely affected by the presence of bacterial biofilms. The surface modification with nanopatterns has been a promising approach for the prevention of bacterial attachment to Ti implant surfaces. Stolzoff et al. [173] used ion beam evaporation and created nanoscale topographical features on Ti surfaces to reduce the bacterial colonization of surfaces. The adhesion of *S. aureus* showed that smaller and more regularly spaced nanofeatures (specifically 40–50 nm tall peaks spaced $\sim 0.25\ \mu\text{m}$ apart) were more effective than surfaces with larger and randomly positioned peaks. In the earlier study of bacterial adhesion on nanofeatured Ti surfaces with different organizations and shapes, it was found that nanorough surfaces produced by electron beam evaporation were less colonized than unmodified Ti surface, while the nanotextured and nanotubular surfaces produced by anodization and acid etching methods were significantly more colonized [174], suggesting that the nanofeatures present on surfaces may affect bacterial adhesion differently depending on their organization and shape. Inspired by the surface pattern of dragonfly wings, Bhadra et al. [175] created the nanowire array patterns on titanium surfaces via hydrothermal etching method and found that the optimal surface structure can increase the adherence of human fibroblasts to substrate, but also increase the bactericidal properties of the surface. The results show that the created nanopatterned surfaces eliminated $\sim 50\%$ of *P. aeruginosa* cells and about 20% of the *S. aureus* cells coming into contact with the surface. These functional surfaces exhibited differential responses to bacterial and human cells and represented the surfaces that have excellent prospects for biomedical applications.

Bacterial adhesion to topographically modified surfaces is a multifactored process. Commonly proposed explanation of results showing the enhanced adhesion or inhibition of bacterial adhesion by surface topography can be from the point of view of surface roughness and availability of surface contact area, wettability, and surface charge. Among them, the effect of surface contact area is the predominant. Regardless of regular or irregular patterned surfaces, the decrease in accessible surface contact area mainly contributes to the reduction of bacterial adhesion. We developed textured polyurethane biomaterial surfaces with the ordered arrays of submicron size pillars, and found that bacterial adhesion was significantly reduced for *S. epidermidis* and *S. aureus* either on hydrophobic or hydrophilic surfaces while

the adhesion was enhanced on the micron-size textured and hydrophilic surfaces since the bacterial cells can access into the spaces between pillars, and accessible surface area increased for micron patterns [176,177]. In the study of surface modification on bacterial adhesion on Ti-based materials, Lorenzetti et al. [178] demonstrated that the effect of surface topography on bacterial attachment was more predominant than the surface wetting and surface charge. When the peak-to-peak distances between surface features were approximately equal to or larger than the bacteria size, the bacterial attachment was favored. In contrast, the structures with fine asperities reduced the contact area between the bacterium and the coating, with consequent lower bacterial adhesion in comparison to the nontreated titanium despite its high hydrophilicity.

The change of surface wettability of topographic surface is another important factor in influencing bacterial adhesion. The micro/nanostructured surface dramatically alters the surface wettability due to the Wenzel or Cassie–Baxter effects, producing the superhydrophobic or superhydrophilic surfaces depending on the wettability of raw surface [179]. In the case of hydrophobic material the trapped air in the spaces between features contributes to the superhydrophobic property of micro/nanostructured surfaces. Due to the small contact area with water, both chemical reactions and bonding formation through water are limited, and the surface exhibits a self-cleaning phenomenon and repels to adhesion [154,180]. Such unique antifouling property of natural surfaces such as taro and lotus leaves, fish scale, or nepenthes pitcher plants are known due to these specific microstructures of surfaces, and have been inspired to design superhydrophobic, underwater superoleophobic, or omniphobic slippery surfaces, respectively, which are effectively to resist proteins, bacteria, cells, and marine organisms [159]. The effect of superhydrophobic surfaces on bacterial adhesion is also reflected on the low drag force for fluid flow. The hydrophobic surface produces a large “slip” at the fluid–solid interface with large reduction in drag force in both laminar and turbulent flows, enhances mixing in laminar flow and amplifies diffusion-osmotic flows [181]. This reduces the gradient of drag forces near the surface and weakens the effect of shear stress on bacterial adhesion. This is probably the reason that we found no significant shear stress dependence of bacterial adhesion seen on hydrophobic textured polyurethane surfaces [177].

In addition to self-cleaning and repellent adhesion properties, nanostructured surfaces also show the bactericidal effect that kills microbes coming in contact with them. The first bactericidal activity based solely on its physical surface structure was found on the natural insect surfaces, for example, the cicada wings. Ivanova et al. [182] reported that *P. aeruginosa* cells were capable of adhering relatively effectively onto the surface of the wings of the Clanger cicada, which consists of nanopillar structures on the wing surfaces (Fig. 2.4A), but those adherent cells were killed with extreme efficiency and quickly (within ~3 minutes) by the wing surface, suggesting the antifouling nature of cicada wings was not due to the ability to repel bacteria, rather to its ability of killing them upon contact (Fig. 2.4B and C). However, the researchers found that the cicada wings were only effectively killing Gram-negative bacteria but not Gram-positive bacteria. This selective killing of Gram-negative bacteria was explained by a biophysical model of the interactions between bacterial cells and cicada wing surface structures. In this model, the cell mechanical properties, in particular cell rigidity, were the key factors in determining bacterial resistance/sensitivity to the bactericidal nature of the wing surface (Figs. 2.4D–G) [183]. As bacterial cells contact and

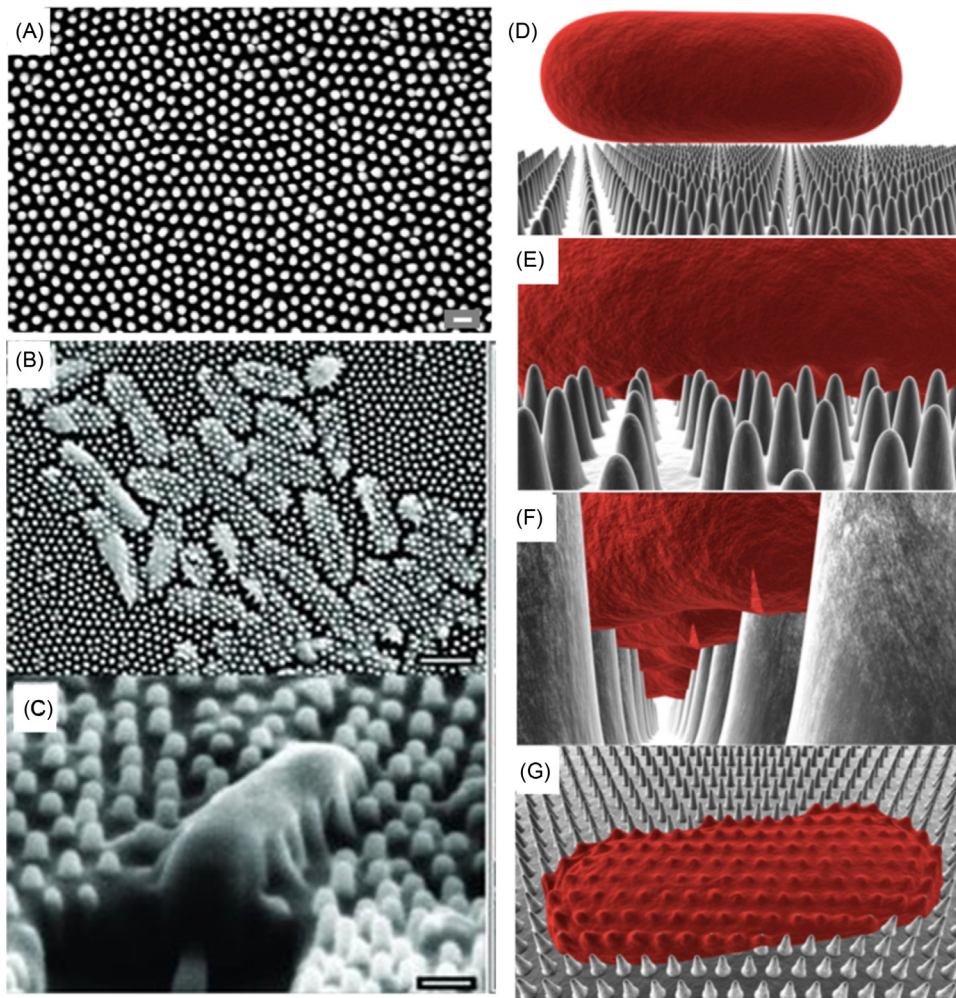


FIGURE 2.4 (A) SEM image of the surface of a cicada wing (scale bar = 200 nm). (B) *Pseudomonas aeruginosa* cells are clearly penetrated by the nanopillar structures on the wing surface. Scale bar = 1 μm. (C) SEM image of a *P. aeruginosa* cell sinking between the nanopillars on the cicada wing surface at 65,000× magnification at an angle of 53 degrees. (D–G) Biophysical model of the interactions between cicada wing nanopillars and bacterial cells. As the cell comes into contact (D) and adsorbs onto the nanopillars (E), the outer layer begins to rupture in the regions between the pillars (F) and collapses onto the surface (G). Source: Reproduced from Ivanova EP, Hasan J, Webb HK, Truong VK, Watson GS, Watson JA, et al. Natural bactericidal surfaces: mechanical rupture of *Pseudomonas aeruginosa* cells by cicada wings. *Small* 2012;8(16):2489–94; Pogodin S, Hasan J, Baulin VA, Webb HK, Truong VK, Phong Nguyen TH, et al. Biophysical model of bacterial cell interactions with nanopatterned cicada wing surfaces. *Biophys J* 2013;104(4):835–40 with permissions from Elsevier, John Wiley and Sons, respectively.

adhere to the nanopillars of cicada wings, the cell membrane stretches in the regions suspended between the pillars, leading to cell membrane rupture if bacterial cell surface is less rigid, for example, Gram-negative strains. However, the rigid Gram-positive cells are resistant to this effect and can survive on surfaces. The bactericidal effect of nanotopography

was also found on other nature surfaces, for example, gecko skin [184] and dragonfly wings [185]. Unlike cicada, the nanostructures present on the dragonfly wings are randomly distributed in terms of shape, size, and distribution. The synthetic black silicon surfaces by biomimicking the nanostructured surfaces of dragonfly wings showed that the black silicon and dragonfly wing surfaces were efficient in killing both Gram-negative and Gram-positive bacteria, and endospores [186]. This bactericidal activity is believed to be driven by mechanical and structural responses to the deformational stresses imposed by the surface nanoarchitecture on the peptidoglycan cell wall and inner membrane of bacterial cell. In the study of bactericidal effects of dragonfly wing's surface on *E. coli*, Bandara et al. [185] demonstrated that the bacterial cell membrane damage was initiated by a combination of strong adhesion between nanopillars and bacterium EPS as well as shear force when immobilized bacterium attempts to move away from the unfavorable surface topography.

The bactericidal effects of these natural surfaces are due to the presence of sharp nanostructures, usually nanopillars. The surface analysis of cicada wings showed that the wings were covered with a periodic topography consisting of hexagonal arrays of spherically capped, conical, nanoscale pillars with 200 nm in height, 100 nm in diameter at the base, 60 nm in diameter at the cap, and 170 nm apart from center to center [182]. The dragonfly wing surfaces consist of a dense array of closely but randomly arranged two prominent populations of nanopillar architecture. These nanopillars are cylindrical in shape with 37 ± 6 nm in diameter and 189 ± 67 nm in height for short pillars or 57 ± 8 nm in diameter and 311 ± 52 nm in height for tall pillars. Tripathy et al. [160] reviewed these natural nanostructured bactericidal surfaces and summarized that the nanopillars shaped with diameter 50–250 nm, height 80–250 nm, and pitch 100–250 nm can pierce into the bacterial cell wall upon contact or rupture the bacteria cell wall, thereby killing the bacteria. Such nanostructured patterns can be biomimicked and applied to the material surfaces to generate a new generation of mechanoresponsive, antibacterial nanomaterials, and have become an attractive approach to potentially tackle multiantibiotic resistant bacteria. The recent advances made in nanofabrication have made it possible to mimic the highly bactericidal topographies found in nature. A variety of nanostructured surfaces have been developed on the silicon [186], titanium [175], and aluminum [187]-based materials as well as polymeric materials [188], which exhibit the bactericidal effects. Linklater et al. [189] reviewed the nanofabrication techniques to produce such mechano-bactericidal surfaces and demonstrated that nanoengineered surfaces, such as carbon nanotubes, graphene and graphene oxide nanosheets, black silicon, nanopillared polymers, and titanium nanowires have all exhibited significant bactericidal activity, enabling their potential use as biomaterials of the future.

2.5 Signaling molecules in the regulation of bacterial adhesion on biomaterial surfaces

When a single bacterial cell is attached on the surface, it will grow, multiply, and form multicellular communities through the numerous interactions with other individual bacterial cells to form aggregates, and finally mature to form complex biofilms on material surface. The transition of attached single cell into biofilm community often triggers important

physiological changes, gene expression, and even morphology for individual cells, causing the generation of an extracellular matrix to protect the cells and strengthen adhesion [190]. The planktonic and sessile bacterial cells may differentiate in gene expression in part associated to the adhesive needs of the population. For example, it was reported that motility genes in sessile species, such as flagella and pili enabling bacteria to reach surfaces and to move along surfaces, are downregulated, and the genes that promote the sessile lifestyle for the production of cell surface proteins and excretion products are upregulated [191]. Signaling molecules have been known crucial for bacterial physiology and pathogenesis, and play an important role in regulating motility and adhesion genes. The best known and well characterized bacterial signaling molecules are autoinducers, which are secreted in response to changes in cell-population density [192]. Bacteria use the quorum sensing systems through these signaling molecules to regulate a diverse array of physiological activities including symbiosis, virulence, competence, conjugation, motility, and biofilm formation [193]. Nucleotide signaling molecules are the other key molecules that help bacteria to rapidly coordinate cellular pathways and adapt to changes in their environment [194]. Among them cyclic dimeric guanosine monophosphate (c-di-GMP) has been recognized as a universal intracellular signaling molecule and plays a central role in coordinating the transition from a motile planktonic lifestyle to a sessile biofilm forming state and vice versa (i.e., dispersal) [195–197].

c-di-GMP is a soluble molecule and functions as second messenger in bacterial cell attachment. It is synthesized from two molecules of GTP by di-guanylate cyclases (DGCs) and is degraded into pGpG and GMP by phosphodiesterases (PDEs) [198,199]. DGCs and PDEs respond to a broad range of environmental cues and modulate intracellular levels of c-di-GMP, which regulate various cellular functions including biofilm formation, virulence, and dispersal in many bacterial species [200–203]. It has been found in several bacterial species, for example, *E. coli* and *P. aeruginosa*, that high c-di-GMP levels in cells were correlated well with biofilm formation or low c-di-GMP concentrations with motility, thereby, c-di-GMP is proposed to stimulate the biosynthesis of adhesins and exopolysaccharide matrix substances in biofilms and inhibits various forms of motility [198]. It is proposed that cells use c-di-GMP as a checkpoint to proceed through the distinct stages of biofilm development until they fully commit to biofilm lifestyle.

The c-di-GMP levels in cells are influenced by the environment. Bacteria can sense various environmental signals, and differentially regulate activity of DGCs and PDEs to coordinately modulate the metabolism of c-di-GMP and shape biofilms to adapt to local conditions. Environmental signals including temperature, light, oxygen, amino acids, growth medium, and growth conditions have been found to regulate c-di-GMP levels and the development of biofilms [204,205]. It is unsurprised that the biomaterial surface properties also influence the c-di-GMP levels and biofilm formation. Rzhepishevskaya et al. [206] studied the *P. aeruginosa* biofilms formation on the antibacterial coating surfaces with varying physicochemical characteristics, and found that a mushroom structured biofilm formed on negatively charged poly (3-sulphopropylmethacrylate) and zwitterionic poly(2-(methacryloyloxy)ethyl)dimethyl-3-sulphopropyl ammonium hydroxide surfaces with high levels of c-di-GMP in cells, while flat biofilms were developed on glass, positively charged poly(2-(methacryloyloxy)-ethyltrimethyl ammonium chloride), protein-repellent poly-oligo(ethyleneglycol methylethermethacrylate) and hydrophobic

polymethylmethacrylate surfaces. The increased level of c-di-GMP in mushroom-structured biofilms suggests that bacteria are capable of a quick physiological response when exposed to surfaces with varying physicochemical characteristics. It was reported that bacteria strains, for example, *E. coli* and *P. aeruginosa*, can sense the stiffness of substrate material during attachment and the decreased stiffness of crosslinked PDMS promoted the adhesion and growth of bacteria [207]. The further study showed that *P. aeruginosa* cells attached on the soft PDMS substrates have higher levels of c-di-GMP compared to stiff PDMS surfaces, suggesting that *P. aeruginosa* responds to such mechanical cues by adjusting c-di-GMP level and the following biofilm formation [208].

Nitric oxide (NO) is an endogenous gas molecule released from the endothelial cells on blood vessels and can effectively prevent the adhesion/activation of platelets on normal blood vessel walls. NO can also serve as an indispensable antimicrobial agent in our immune response system to combat infectious diseases. NO-releasing biomaterials have represented an important biomimetic strategy for antithrombus and antimicrobial infections with great potential for clinical use [209,210]. One mechanism of NO to control biofilm infection is that NO, as a diatomic free radical, can cross the membranes to enter the microbial cell readily and kill the microbe by directly damaging DNA, proteins, and lipids through production of potent nitrosating species or by combining with reactive oxygen species (e.g., superoxide, peroxide) and oxidizing the same targets [211,212]. The other mechanism is that NO can serve as a signaling molecule and trigger biofilm dispersal at low, nontoxic concentrations [213]. The study further showed that NO signaling in *P. aeruginosa* biofilms mediated PDE activity, decreased c-di-GMP levels, and enhanced dispersal. The gene expression studies indicated upregulation of genes involved in motility and energy metabolism and downregulation of adhesins and virulence factors responding to NO in *P. aeruginosa* biofilms [214].

The link between c-di-GMP signaling and biofilm formation affords novel strategies for treatment of biofilm-associated infections whereby modulation of the levels of the nucleotide or interference with signaling pathways may lead to inhibition of biofilm formation or promotion of biofilm dispersal [215]. The c-di-GMP signaling inhibitors that reduce the intracellular c-di-GMP level via diguanylate cyclase inhibition or PDE activation will control the biofilms formation. Christensen et al. [216] demonstrated *in vitro* and *in vivo* that the induction of the *E. coli* YhjH c-di-GMP PDE can lead to dispersal of the *P. aeruginosa* biofilms. Ma et al. proposed protein engineering to evolve amino acid BdcA which can greatly binding c-di-GMP and decrease the concentrations of c-di-GMP, resulting in the complete removal of biofilms via dispersal without affecting initial biofilm formation [217]. These works prove the concept that interference with c-di-GMP signaling is a promising strategy for treatment of biofilm-based infections. Karaolis et al. [218] proposed to use extracellular c-di-GMP against the activity of *S. aureus*. The synthesized c-di-GMP can be used either alone or in combination with other antimicrobial agents to inhibit the cell-cell interaction of *S. aureus* and biofilm formation, providing an alternative approach to the prevention of biofilms and the treatment of infection.

Other cyclic nucleotide second messengers, for example, cyclic adenosine-monophosphate (cAMP), cyclic guanosine-monophosphate, and cyclic dimeric adenosine monophosphate, are also crucial in the transduction of signals and have been found to be involved in regulation of motility and adhesion [219]. In the study of biofilm formation by *P. aeruginosa* PAO1,

cAMP was found to regulate the attachment of cells by inhibition of the transition from reversible to irreversible attachment, which is the first step of biofilm formation. Further analyses revealed that cAMP altered the cell surface hydrophobicity thereby inhibiting cell's attachment to the surface [220]. The latest study shows that the increased levels of cAMP inhibit *P. aeruginosa* biofilm formation through the reduction of the c-di-GMP levels as well as the reduction of biofilm matrix components [221]. Cyclic di-AMP is an essential molecule in the signaling pathways that regulate the viability and virulence of bacteria [222]. It also regulates the biofilm formation through enhancing the production of polysaccharides. Similar as c-di-GMP, the level of c-di-AMP is modulated by activity of di-adenylyl cyclase that produces c-di-AMP and PDE that degrades c-di-AMP. In the study of biofilm formation by *Streptococcus mutans*, Peng et al. [223] found that the increased c-di-AMP levels by deletion of the *pdeA* gene coding for a PDE promoted biofilm formation. The c-di-AMP-mediated biofilm enhancement was found to be due to the upregulating production of glucans, a key component of biofilm matrix. However, the accumulation of c-di-AMP does not always promote biofilm formation. In *B. subtilis*, c-di-AMP is essential for the growth of cells, but a high concentration of c-di-AMP is toxic. Gundlach et al. [224] found that the deletion of two PDEs of c-di-AMP in *B. subtilis* growth caused the accumulation of c-di-AMP and inhibited biofilm formation.

2.6 Summary and perspectives

A larger number of research works have been done and tremendous progress has been made in understanding the bacterial interactions with biomaterials and biofilm formation in past decades. Most of this new knowledge has been based on the elucidation of genetic pathways, physiological responses, and intracellular signal transduction pathways, such as those that are regulated by c-di-GMP, c-di-AMP, and cAMP [190]. However, due to the complicated interactions occurred between the cells, biomaterial surfaces, and environments, it is hard to draw the general conclusions on how adhesion is achieved. The bacterial cell, the liquid environment, and material surface itself are dynamic, and the change of one can have a major effect on each other and cause different responses of cells and interactions. The future studies will need to address how the bacterial cells sense environmental cues and how the production and variability of extracellular molecules (EPS) of bacteria are regulated, as well as how bacteria use the signaling molecules to adapt to the environment and regulate bacterial adhesion and biofilm development. For example, surface contact-stimulated adhesion production is important for bacterial adhesion and biofilm formation, and it has been known to be triggered by surface sensing through the cell envelope or extracellular appendages, however, the underlying molecular mechanisms are not yet fully understood [225]. Biofilm formation is initiated from the individual cells attachment and colonization, thus the understanding of bacterial behaviors at single bacterial cell level would be important for a better understanding of how multifactorial parameters influence initial adhesion. The development of new techniques or an improvement of the existing techniques will help to understand the single cell level of bacterial responses to material surface and environment. For example, the new molecular biology techniques that allow proteomic and genomic studies on single bacterial cells and the

microscopy techniques including AFM, confocal laser scanning microscopy, fluorescence microscopy, and electron microscopy are bringing about a significant increase in understanding of single cell responses and biofilm formation on material surfaces [226].

It is well established that implanted biomaterials are immediately adsorbed and coated with a layer of proteins from blood or interstitial fluid, and it is through the protein layer that the cells sense foreign surfaces. The conformational structure and biological function of proteins adsorbed are affected by material surface properties (e.g., surface chemistry, nanotopography) and they in turn influence bacterial adhesion. Understanding the molecular interaction of proteins at interface of material-bacteria is fundamental to understand the bacterial adhesion to biomaterial surfaces. Future research in this field will need to better address the correlation of surface properties and protein conformation/functions, and correlate them to the biological response of bacterial adhesion, especially at the nanoscale molecular level. For example, proteins are particularly sensitively to nanotopography of surface, the development of nanobiomimicking biomaterial surfaces consisting of nanoscale topography will largely rely on a better analysis and understanding of the influence of nanostructure on the quantity and conformation of proteins adsorbed on surfaces.

While the new knowledge and mechanisms of bacterial responses to material surfaces and environments are revealed, the number of antibacterial surfaces is also quickly expanding. Antibacterial surfaces are capable of repelling bacterial cells and preventing their attachment, or inactivating/killing cells upon contacting with the surfaces. The current research in antibacterial and bactericidal mechanisms and models may provide an excellent starting point in understanding the mechanisms of biological responses to these surfaces, however, the additional biological parameters, for example, bacterial cell surface structure, staining type, bacteria dynamics, and mechanical properties, need to be explored and integrated into the current knowledge. Furthermore, greater consideration should also be given to studying the biocompatibility of new biomaterials or new techniques, which can maximize the efficacy in controlling the microbial infections, but also minimize the toxicity and blood responses such as thrombosis.

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Macrophage response to biomaterials

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3.1 The macrophage

Macrophages were discovered in 1884 by Ilja Iljitsch Metschnikow (also written as Élie Metchnikoff), a Russian zoologist best known for his pioneering research in immunology and also considered the father of cellular immunology. He and Paul Ehrlich were jointly awarded the Nobel Prize in Physiology or Medicine in 1908 “in recognition of their work on immunity.” Macrophages were named after their capability to engulf and digest cellular debris or foreign substances. Metchnikoff hence referred to these cells as “the big eaters” in Greek. The term macrophage is formed by the combination of the Greek terms “makro” meaning big and “phagein” meaning eat [1,2].

In terms of their origin, macrophages have been considered for a long time as constituents of the mononuclear phagocyte system. This system includes highly phagocytic cells whose progenitors arise from the bone marrow and enter tissues from the circulation and afterwards differentiate into the different types of tissue macrophages. However, recent studies revealed that not all macrophages are derived from blood circulating monocytes. Macrophages can be derived from primitive macrophage precursors in the embryonic yolk sac and give rise to most types of tissue-resident macrophages. Thus, most adult tissue-resident macrophages are seeded before birth, have self-renewal capacity, and are maintained independently of monocytes. Macrophages from both lineages coexist in many tissues, and either perform redundant, overlapping functions or are capable of a functional collaboration [3–5].

In terms of their function, these immune cells are specialized in the phagocytosis and neutralization of cellular debris and potentially hazardous agents, including pathogens. Tissue macrophages are nonmigratory cells that monitor their immediate, local environment [6]. Moreover, macrophages have a key role in orchestrating the inflammatory reaction by

delivery of chemokines and cytokines, which recruit and activate neutrophils, monocytes, and lymphocytes. In response to an inflammatory stimulus, these cells also produce large amounts of effector molecules, including several growth factors, such as for example platelet-derived growth factors and fibroblast growth factors [7].

In order to accomplish their role, macrophages are prepared with a vast array of sensing molecules that include scavenger receptors, pattern recognition receptors [Toll-like receptors (TLRs), C-type lectin receptors, RIG-I-like receptors, nod-like receptors], cytokine receptors, and adhesion molecules [3].

3.2 Macrophage plasticity and polarization

Macrophages have a central role in inflammation and host defense, they are a crucial component of innate immunity. In addition, these cells also have homeostatic functions that include tissue remodeling in ontogenesis and orchestration of metabolic functions [8].

Macrophages are outstanding plastic cells that can shift from one phenotype to another. In tissues, mononuclear phagocytes respond to environmental cues (such as microbial products or damaged cells) with the acquisition of distinct functional phenotypes. In response to different cues, macrophages may undergo classical M1 activation [stimulated by TLR ligands and interferon (IFN- γ)] or alternative M2 activation [stimulated by interleukin (IL)-4/IL-13] [8,9]. This phenomenon of the two different M1/M2 phenotypes is referred to as “macrophage polarization” and mirrors the Th1–Th2 polarization of T cells. The local cytokine milieu can orientate macrophage polarization. Thus, several classes of macrophages have been described in humans and mice based on the expression of their cell surface markers, production of specific factors, and biological activities [10–12]. M2 macrophages can additionally be divided into three different subsets: M2a, M2b, and M2c. M2a are induced by IL-4 or IL-13; M2b are induced upon exposure to immune complexes and agonists of TLRs or IL-1r, and M2c by IL-10 and glucocorticoid hormones [11].

Macrophage polarization represents a continuum, being that the M1 and M2 are fully polarized macrophages at the extremes. At any given time point the macrophage population will show a combination of macrophage phenotypes and/or macrophages in a state of transition that express markers of both M1 and M2 phenotypes. Thus, a balance in macrophage phenotypes plays a key role in homeostasis and pathogenesis [13,14] (Fig. 3.1).

Different macrophage populations can be generated according to distinct immune signals: (1) *Classically activated macrophages* are effector macrophages that present enhanced microbicidal or tumoricidal capacity and have the ability to secrete high levels of pro-inflammatory cytokines and mediators. These macrophages differentiate in response to IFN- γ and tumor-necrosis factor (TNF). These macrophages are rather important components of the host response, but their activation must be closely controlled because they can lead to host-tissue damage caused by the high levels of pro-inflammatory cytokines and mediators that they produce; (2) *Wound-healing macrophages* are developed in the presence of IL-4. These macrophages produce minimal amounts of pro-inflammatory cytokines and are less efficient than classically activated macrophages at producing reactive species and at killing intracellular pathogens. Nevertheless, they produce components of the extracellular matrix (ECM) and thus their primary role seems to be related to wound healing.

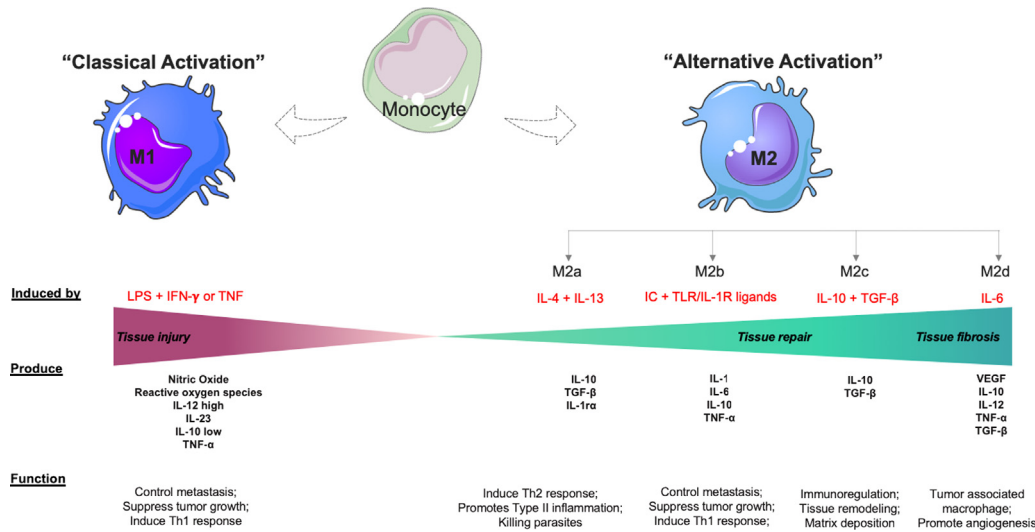


FIGURE 3.1 Spectrum of macrophages activation and polarization and its role on tissue repair. M1 macrophages or M(IFN-γ), or classically activated macrophages, are highly phagocytic, produce large amounts of pro-inflammatory mediators, and promote Th1 response. M2 macrophages or M(IL-4), or alternatively activated macrophages, are antiinflammatory and support angiogenesis and tissue repair. M2 macrophages produce large amounts of antiinflammatory mediators and promote Th2 response. M2 macrophages have different subsets, induced by different molecules and different activation responses. M2a macrophages or M(IL-4 or IL-13) are pro-fibrotic and mainly induce a Th2 response. M2b or M(IC or TLR/IL1-R ligands) macrophages are also involved in Th2 activation and immune regulation, being referred to as regulators. M2c or M(IL-10 or TGF-β), are involved in immune suppression, tissue repair, and remodeling. M2d or tumor-associated macrophages exhibit functions associated to tumor progression inducing new blood vessel growth. *IFN*, Interferon; *IL*, interleukin; *TLR*, Toll-like receptor. Source: Adapted from Abcouwer SF. Angiogenic factors and cytokines in diabetic retinopathy. *J Clin Cell Immunol* 2013;Suppl 1 [55].

These macrophages if their activity is dysregulated can be harmful to the host because they can lead to the formation of tissue fibrosis, and (3) *regulatory macrophages* have the important role of reducing immune responses and thus limiting inflammation. The production of the regulatory cytokine transforming growth factor (TGF)-β by macrophages following phagocytosis of apoptotic cells can contribute to the generation of this type of macrophage. This type of macrophage produces high levels of the immunosuppressive cytokine IL-10 and does not contribute to the production of ECM as wound-healing macrophages. A dysregulation in the activity of these macrophages can predispose the host to infection [15–17].

3.3 The macrophage response to biomaterials

The process of implantation of a biomaterial will lead to an inflammatory response that is caused by the injury made through the surgical procedure and also by the presence of the implanted material. This response can be divided into different phases starting with

(1) blood–material interactions (2) release of danger signals by injured cells, (3) acute inflammation, (4) chronic inflammation, and (5) foreign body reaction [18,19]. The macrophage is the dominating infiltrating cell in response to biomaterial implantation and has an important role in the different steps of the inflammatory response as well as in the subsequent tissue repair/regeneration. In addition, at the surface of the material, macrophages can present a morphologic variant since they may fuse and form foreign body giant cells (FBGCs) [20].

Macrophages are considered as the orchestrators of the inflammatory response to biomaterials because they have an important role in all steps of this response. We will briefly review the role of macrophages in each one of the inflammatory response phases enumerated above:

1. Almost immediately after biomaterial implantation, several different proteins (such as coagulation, complement, and adhesion proteins) from blood and interstitial fluids adsorb to the surface of the material [18]. It is suggested that phagocytes, namely macrophages, sense some of the adsorbed proteins, such as complement proteins and fibrinogen, and thus an inflammatory response is triggered [21]. Additionally, adhesion proteins of the ECM such as fibronectin and vitronectin are known to promote the formation of FBGCs at the surface of the material [22,23].
2. The implantation of a biomaterial causes injury to cells at the implant site. These injured cells will release danger signals, generally designated as “alarmins” that act as a potent activator of cells of the innate immune system as macrophages causing their priming and activation. Alarmins are the endogenous equivalent of pathogen-associated molecular patterns including for example heat shock proteins, ATP, and uric acid [24–26].
3. The acute inflammatory phase is dominated by the presence of polymorphonuclear leukocytes (PMNs). Nevertheless, macrophages also have a role in this phase since the activated PMNs will secrete several chemokines such as monocyte chemoattractant protein (MCP-1) and macrophage inflammatory protein (MIP-1 β) which are chemoattractants and activators of monocytes and macrophages [17,26,27].
4. Chronic inflammation may develop if the inflammatory stimuli persist; and the macrophage is a fundamental cell type of this phase of the inflammatory response. Macrophages release a great number of biological active inflammatory mediators such as TNF- α , IL-8, IL-1 β , MCP-1, and MIP-1 β among others that will recruit further inflammatory cells [28,29].

The last phase of the inflammatory response, the foreign body reaction, is clearly dominated by the action of macrophages. Since macrophages can only phagocytose particles up to 5 μm , in the presence of larger biomaterials they will fuse and form BGCs. Studies suggest that after fusing in FBGCs, a reduction in the phagocytic activity is observed together with a higher degradative capacity caused by the release of reactive species, thus generating an extremely degradative environment at the biomaterial surface. In addition, FBGCs secrete fibroblast recruiting factors which will lead to fibrous encapsulation around the biomaterial due to collagen deposition. This fibrous capsule will impair the implant function isolating it from the local tissue environment [17,20,30,31].

Being an important cell type of the immune system, the macrophage has important functions related to immune responses, inflammation, and foreign body responses.

However, additionally macrophages are very important in tissue repair since they have a significant role in the regulation and recruitment, proliferation, and differentiation of cells such as fibroblasts, osteoblasts, and keratinocytes among others [20,32].

A more in depth understanding of the rather complex interactions between macrophages and biomaterials is key for the development of biomaterials and biomedical devices, and also to improve biomaterial/tissue integration and biomaterial function.

3.4 The macrophages and the development of immunomodulatory biomaterials

3.4.1 Immunomodulatory biomaterials

The discovery that immune mediators have a key role in repair and regeneration is now considered in the development of biomaterial scaffolds that have the ability to modulate the immune response. An immunomodulatory biomaterial can be described as material that is able to modulate immune signaling and thus create a pro-regenerative environment. This concept leads to the appearance of the new concept of biomaterials-directed regenerative immunology [33].

These immunomodulatory biomaterials are expected to influence immune cell function stimulating tissue healing together with the integration of the implant while supporting its function [34]. There are different strategies used in biomaterial-based immunomodulation such as (1) altering the physical properties of the materials; (2) tuning of the chemical properties of biomaterials; (3) combination of bioactive molecules either antiinflammatory drugs or pro-resolution mediators or growth factors; (4) biomaterials based on decellularized ECM; and (5) cell therapy methods that can either include immune cells or can induce their recruitment [35] (Fig. 3.2).

Biomaterial-based immunomodulation strategies have the potential to improve the outcomes of biomedical implants and tissue engineering therapies. The idea is to modify the biomaterial in such a way that a microenvironment that controls the inflammatory response and promotes tissue repair is created [35].

The field of research related with immunomodulation and biomaterials is vast and includes different research areas such as cancer immunotherapy, vaccination, establishing tolerance in organ transplantation and treatment of autoimmune disorders [36]. However, herein we will focus on immune-mediated strategies for tissue engineering and regenerative medicine.

3.4.2 Macrophages in immunomodulation

The studies described in the literature attempting to modulate immune responses are mostly focused on the macrophage, namely in macrophage polarization. This is because macrophages are highly plastic cells [8,10] that play a decisive role in inflammation and also in the coordination of tissue repair, fibrosis, and tissue regeneration [37]. The remarkable plasticity of macrophages is making them a rather interesting target for immunomodulation. However, this remarkable functional plasticity can play both positive and

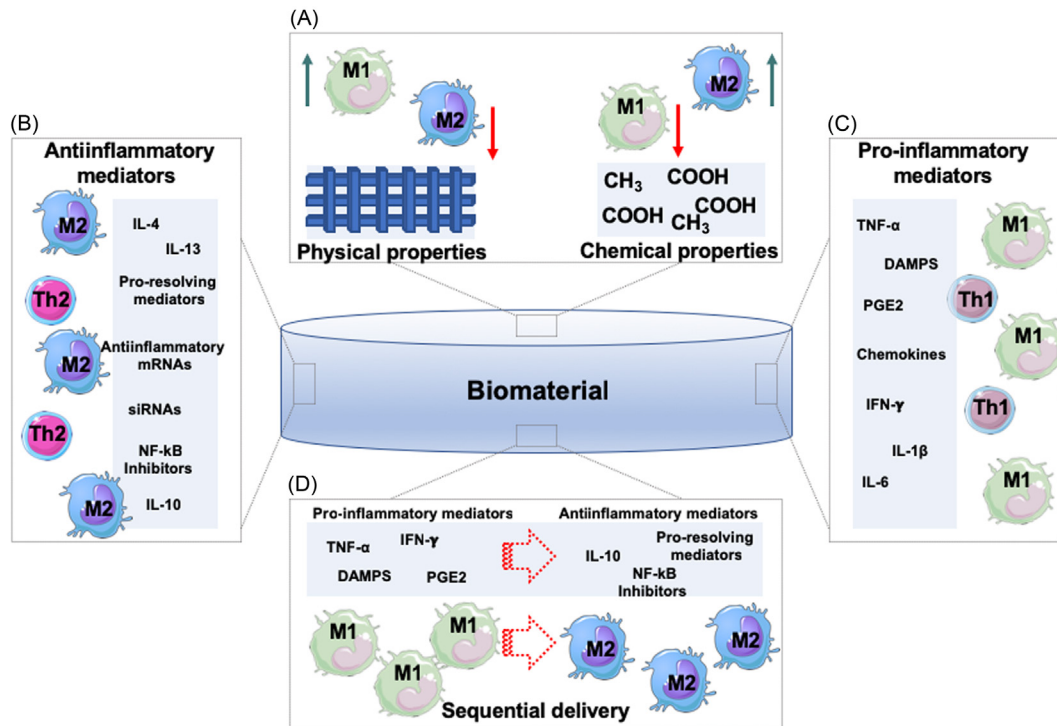


FIGURE 3.2 Approaches based on immunomodulatory materials to promote tissue repair. Different strategies could be used to improve the repair process through the modulation of the immune cells (macrophages). (A) The physicochemical properties of the material could be changed in order to obtain a desired immune response. (B, C) The material itself could be used to delivery immune mediators (pro or antiinflammatory) in order to achieve different responses, being the most common strategy. (D) The most interesting and complicated approach is the sequential delivery of pro and antiinflammatory mediators to apply a more accurate control over the tissue healing. The sequential release aims to induce an effective regenerative microenvironment. Different stages of tissue repair must be carefully regulated, with macrophages of different phenotypes playing unique and critical roles at each stage. M1 macrophages are indispensable for the initiation of the regeneration process being substituted by M2 macrophages which are essential for the resolution of inflammatory response and tissue homeostasis. Source: Adapted from Julier Z, Park AJ, Briquez PS, Martino MM. Promoting tissue regeneration by modulating the immune system. *Acta Biomater* 2017;53:13–28 [56].

negative roles in the pathogenesis of disease and in tissue remodeling, and must thus be well balanced.

An appropriate host immune response that includes a timed transition in macrophage phenotypic profile from pro-inflammatory (M1-like) to pro-remodeling (M2-like) is associated with a downstream constructive remodeling. In addition, the resolution of the pro-inflammatory microenvironment is needed for an effective clinical outcome following the use of any biomaterial [38,39].

The macrophage population present closely following tissue injury retains predominantly M1 characteristics. The alteration to an M2 phenotype occurs concomitantly with the resolution of the inflammatory response and the initiation of the remodeling phase of

wound healing. Several results reported in the literature support the idea that phenotypic differences in macrophages are associated with distinct tissue remodeling outcomes. In fact, higher ratios of M2:M1 macrophages are associated with improved outcomes [14,40–42].

The evidence that M1 macrophages appear at the early stages of normal wound healing and that are followed by M2 macrophages, suggests that a biomaterial that initially promotes an M1-like response followed by a conversion to a M2 phenotype would achieve better outcomes in terms of biomaterial integration and subsequent tissue repair. Following this idea, Spiller et al. [42] developed a strategy to be applied in bone regeneration biomaterial using modified decellularized bone to create a scaffold that allows an initial short release of IFN- γ that will induce M1 macrophage polarization, followed by a sustained release of IL-4 to promote a shift towards the M2 phenotype. Using the same justification, Chen et al. [43] developed a biomaterial consisting of a double hydrogel layer on titania nanotubes to achieve a controlled release of IL-4 and IFN- γ . Alhamdi et al. [44] established a novel drug delivery system for temporal guidance of the polarization of macrophages using bone grafting materials. A biomimetic calcium phosphate coating (bCaP) physically and temporally separated the pro-inflammatory stimulus IFN- γ from the pro-reparative stimulus simvastatin. They were able to induce a sequential M1-to-M2 activation suggesting that this novel immunomodulatory drug delivery system holds potential for controlling macrophage activation in bone repair. We have developed an immunomodulatory strategy, also based on the idea of inducing a shift in the macrophage phenotypic profile. For that we have used 3D porous chitosan scaffolds with a 15% degree of acetylation that induce a typical pro-inflammatory response [45] together with the delivery of specialized pro-resolution mediators, namely lipoxin A4 and resolvin D1, and we were able to in vivo shift the macrophage phenotypic profile towards a M2 reparative response [46,47]. Kumar et al. [48] have developed a rapid self-gelling silk-based injectable hydrogel. This silk gelation behavior, in mild encapsulation conditions, facilitates the incorporation of islets and biomolecules (drug/growth factor) without compromising their biological activities. They have prepared IL-4 (antiinflammatory cytokine) and dexamethasone (immunosuppressive drug) -loaded silk-blended hydrogels to investigate the versatility of these hydrogels for islet encapsulation and biological functionalization (immunomodulation and endothelialisation). These hydrogels showed the capacity of inducing localized polarization of macrophages towards antiinflammatory macrophages (M2) showing immunomodulatory and immunosuppressive effects which may favor the implant function and stability in vivo. Wang et al. [49] developed a photoreponsive 3D hyaluronan nanocomposite hydrogel that provides control over the activation of integrin-mediated ECM adhesion sites for macrophage immunomodulation over time. By leveraging photoresponsive nanocomposites, they provided an on demand and dynamic 3D hydrogel system that defines the dosage and kinetics of RGD adhesive peptide conjugation to ECM, and their results highlighted that periodic $\alpha\text{v}\beta\text{3}$ integrin activation for 72 hours is critical for sustaining an antiinflammatory M2 phenotype. They state that the implications of this temporal control over macrophage immunomodulation represents a novel strategy to control inflammatory responses that may accelerate endogenous tissue repair responses. Shayan et al. [50] have used nanopatterned bulk metallic glasses to modulate murine macrophage polarization and concluded that nanopatterned surfaces

lead to a more constructive tissue repair with higher vascularization and increased M2 to M1 ratio, when compared to flat surfaces. Wang et al. [51] have produced macroporous electrospun polycaprolactone scaffolds with different fiber sizes and concluded that macrophages cultured on thicker-fiber scaffolds tended to polarize into M2 phenotype, whereas those cultured on thinner-fiber scaffolds expressed mainly M1 phenotype. Lee et al. [52] have performed a chemical surface modification in titanium implants using the divalent cations calcium and strontium and were able to upregulate M2 macrophage phenotype expression. Li et al. [53] have developed titanium implants doped with magnesium with the objective of assessing the macrophage response both *in vitro* and *in vivo* and were able to induce a higher percentage of M2 macrophages and higher concentrations of the antiinflammatory cytokines IL-4 and IL-10.

Several other studies related to tissue engineering and regenerative medicine applications involving M1–M2 immunomodulation can be found. Researchers of this area of investigation aim to accomplish a small pro-inflammatory period in which M1 macrophages are recruited to the site, followed by an antiinflammatory stage where the M2 phenotype dominates. Thus, development of new and smarter materials for M1–M2 modulation is clearly an area in development that will continue to receive significant attention [54].

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Dendritic cells responses to biomaterials

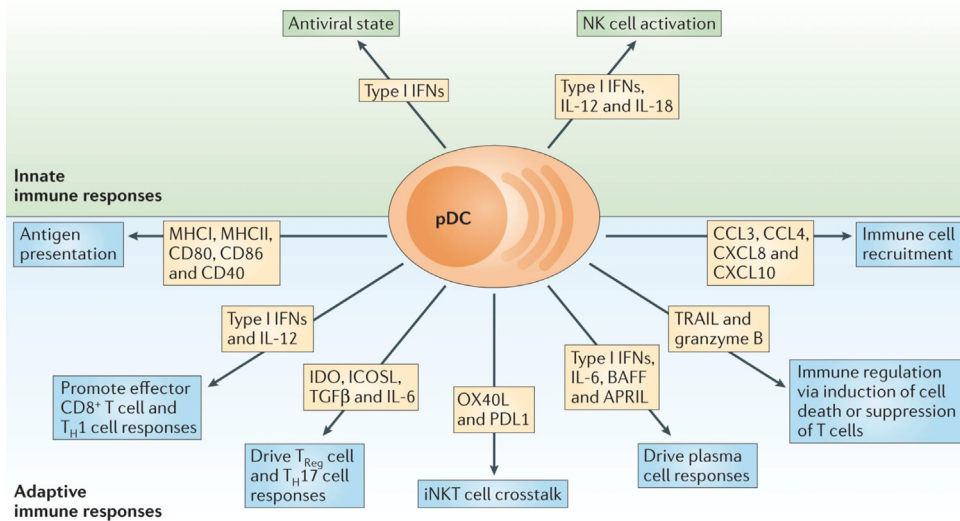
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4.1 Introduction

Dendritic cells (DCs) are antigen presenting cells (APCs) that form the bridge between innate and adaptive immunity [1]. These cells were discovered by Steinman and Cohn in 1973 during a microscopic study of the glass-adhering mouse splenocytes [2]. They were named DCs due to their unique morphology [3]. DCs have multiple subpopulations and are a heterogenous group of cells [4,5]. Of the numerous DC subsets identified to date, each displays the ability to carry out specific functions, a unique phenotype, and the property to enable adaptive immune response [6]. DCs have been extensively characterized owing to their distinct set of cell-surface markers [7,8] (Fig. 4.1).

DCs originate from hematopoietic stem cells in bone marrow [8]. There is much debate about hematopoietic stem cells' role to instruct lineage differentiation or lineage specification of DCs [10]. The development and homeostasis of DC depends on several cytokines, namely, Flt3L, Granulocyte macrophage – colony stimulating factor (GM-CSF), and lymphotoxin- β . Initially discovered on committed lymphoid precursors and stem cells, FMS-like tyrosine kinase 3 (Flt3) is a tyrosine kinase receptor [11]. Its deficiency causes loss of myeloid DCs (cDCs) and plasmacytoid DCs (pDCs) in the lymph nodes, spleen, and surrounding tissue [12]. GM-CSF plays a multifold role in the development of DCs. They regulate homeostasis and the function of resident DCs in nonlymphoid tissues [13]. GM-CSF promotes differentiation of circulating monocytes and hematopoietic progenitors into DC morphology resembling cells, capacity to promote T lymphocytes and cell surface marker expression [14,15]. Using a prolonged culture combining Flt3L and GM-CSF, efficient production of CD103⁺ DCs in bulk has been reported [16]. In addition to these, lymphotoxin- β [17] and macrophage – colony stimulating factor [13] also play a crucial role in DCs development. These DCs play an important role in modifying adaptive immune system.



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FIGURE 4.1 pDCs are an important driving force of the innate and adaptive immune system. The figure outlines the various functions carried out by pDCs in the biological system through presentation of multiple proteins [9]. *pDCs*, Plasmacytoid dendritic cells.

Several DC progenitors have been studied in mice as well as humans. A macrophage-DC progenitor (MDP) which has lost its granulocyte potential has been identified in mice [18]. MDPs are $\text{Lin}^{-}\text{Sca}^{-}\text{Kit}^{\text{hi}}\text{Flt3}^{+}\text{CD115}^{+}\text{CX3CR1}^{+}$ cells that can produce DCs, macrophages, and monocytes. However, they have been found to lack megakaryocyte, lymphoid, and granulocyte potential. An important pathway in DC hematopoiesis is represented by MDPs. They aid in bridging the gap between the innate and adaptive immune response by acting as a specialized progenitor to produce mononuclear phagocyte system cells [19]. A common dendritic progenitor (CDP) is produced by the MDPs along the DC pathway. The CDPs lose their monocyte potential and bifurcate into pathways producing pDC and cDC while retaining Flt3 and M-CSFR respectively [20,21].

There are many similarities encountered in the development of DCs in mice and humans. To further study the DC progenitors in humans, a culture system at the population and single cell level was established [22]. This helped in the analysis of differentiation of human granulocytes, lymphoid cells, monocytes, and DCs at the same time [23]. Further clonal analysis of granulocyte monocyte progenitors (GMPs) using the culture resulted in loss of monocyte and granulocyte potential for some GMPs [22]. The heterogeneity of clonal potential was observed prompting fractionation of the GMP population. Three distinct subpopulations were isolated within GMPs with DC potential using cytokine receptor expression. One population consisted of monocytes, granulocytes, and DC potential (hGMDP). The second population contained a downstream of hGMDPs that produce only monocytes and DCs but lack granulocyte potential (hMDP).

The third population emerged from hMDP and produced only DCs while lacking monocyte and granulocyte potential (hCDP). Hpre-cDCs is an assigned precursor of cDCs that is located in the bone marrow, blood, and other peripheral lymphoid organs and were found to be produced by hCDPs [24]. Therefore in both mice and humans, the development of DC lineage is a process of increasing commitment through sequential loss of potential to granulocyte, monocyte, and other DC subsets.

Many studies have been performed to understand the physiological functions of DCs [25–27]. Interestingly, studies have indicated that mature DCs express increasing levels of major histocompatibility complex II (MHC II) and are uniquely potent in stimulating T-cell (lymphocytes) proliferation [28,29]. Moreover, DCs are unique among APCs, because their antigen capturing and induction of specific T-cell responses can be two orders of magnitude higher than other APCs [28]. DCs are also known to initiate T-cell immunity and tolerance by regulating their differentiation toward various subsets, namely Th1, Th2, Th17, or T_{reg} [30]. In addition to T-cells, DCs can also indirectly skew the differentiation of innate lymphoid cells (ILCs). These ILCs are known to produce cytokines that modulate the function of T-cells thus initiating adaptive immunity [31].

In this manner, DCs play an important role in initiating both innate and adaptive immune responses, which is relevant in several biomedical applications, such as biomaterial implants, tissue engineering, drug delivery, among others. For example, when there is a wound (due to implants) DCs infiltrate the tissue at the site of injury. One of the main functions of DCs is to generate an innate immune response at the site of biomaterial implants, microparticles, or nanoparticles injections, and at the site of infiltration of foreign materials. Moreover, DCs are also responsible for generating an adaptive immune response at the lymph nodes, once the foreign material has been phagocytosed and processed by the DCs. Therefore in order to modulate DC responses in vivo it is important to design biomaterials introduced in the body (Fig. 4.2).

Lately, there has been a growing interest in the field of biomaterial-based immunomodulation [33–37]. For instance, a strong antigen specific immune response is required to develop an effective cancer immunotherapy strategy by delivering tumor antigens to APCs, more importantly to DCs [38]. Biomaterials have been utilized for numerous biomedical applications to induce a strong immune response [39].

4.2 Natural polymer biomaterials

Natural biomaterials are abundant in nature and resemble the constituents in the biological extracellular matrices [40]. These biomaterials are easily accepted by the body and are known to possess a high bioactivity [41,42]. Natural polymers are biocompatible and easily biodegradable, that is, its products after degradation do not cause any cytotoxicity in the body [43,44]. Some of the commonly used natural biomaterials are chitosan, gelatin, alginate, and hyaluronic acid among others.

4.3 Gelatin

Gelatin is a natural protein that can be obtained from the hydrolysis of collagen. It is easily soluble in water at 37°C [45] and exhibits amphoteric behavior [46]. Gelatin can readily

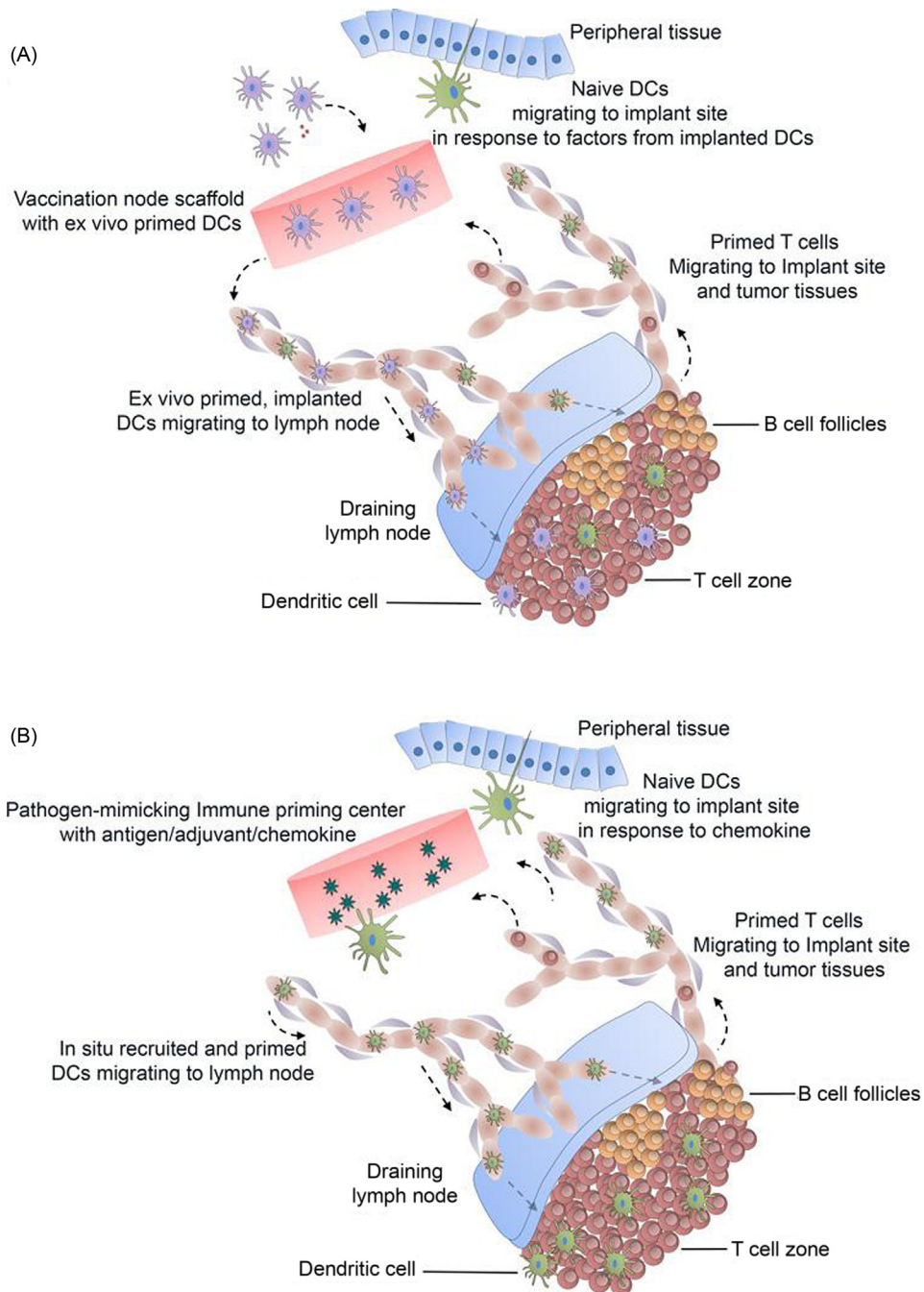


FIGURE 4.2 A schematic representation of DC trafficking and modulation using biomaterials is illustrated [32]. (A) Ex vivo primed DCs are delivered s.c. into mice through implants or injections. Some of these primed DCs traffic toward the draining lymph nodes and its present antigen to T lymphocytes. The chemokines and cytokines released by these DCs at the site of injected/implant recruit host naïve DCs and the programmed T lymphocytes to induce a strong immune response. (B) To attract naïve DCs, numerous chemokines and growth factors are released in the tissue upon in situ crosslinked biomaterials. Upon traveling to the draining lymph nodes, these recruited DCs phagocytose antigenic vaccines and presented the antigen to naïve Th cells (CD4 + T cells). These T lymphocytes then traffic toward the injection or tumor site to kill the hostile cells. DC, Dendritic cell; s.c., subcutaneous.

crosslink with different molecules exhibiting varied chemical and physical properties for specific applications [47–49]. Therefore because of its biocompatible and biodegradable nature including its readiness for hydrogels, gelatin has garnered a lot of attention in the biomedical field [50,51].

In one study, nanoparticles were made out of gelatin to test the quantitative and qualitative uptake in murine DCs [52]. The main objective of the study was to develop an effective gelatin-based nanoscale vaccine delivery system. Another goal of the study was to successfully deliver tetramethylrhodamine conjugated dextran (TMR-dextran), using the gelatin carrier system as a model drug into DCs. TMR-dextran was integrated in the gelatin-based nanoparticles (GNPs) during its preparation. Flow cytometry analysis revealed that 88% of the specific murine DC marker CD11c cells took up the TMR-dextran loaded GNPs while only 4% of the soluble TMR-dextran form was taken up. Using double color confocal laser scanning microscopy (CLSM), it was seen that DCs phagocytosed the GNPs and the triple color CLSM indicated the localization of TMR-dextran mainly in lysosomes. Release studies *in vitro* by degradation of GNPs using trypsin resulted in release of 80% of TMR-dextran while release efficacy of TMR-dextran integrated GNPs in PBS was minimal. Therefore the study successfully shows the potential of GNPs in cancer immunotherapy as a biocompatible delivery vehicle to DCs.

In another study, these cationic GNPs were utilized as vehicles to enhance the CpG oligonucleotides (CpG ODN) delivery efficacy [53]. Researchers monitored the uptake and their immune stimulatory response of these CpG ODN-loaded gelatin nanoparticles (CpG-GNPs) into murine myeloid DCs. It was observed that, in the murine system, there was an upregulation in uptake and immunostimulatory activity of CpG ODN when CpG-GNPs were delivered both *in vitro* as well as *in vivo*. Interestingly, an enhanced production of interferon (IFN)- α , an essential cytokine in driving both the innate and adaptive immune responses was seen upon delivery of these CpG-GNPs to primary human pDCs. Hence, the study shows the use of GNPs as a biodegradable and well efficacious CpG ODN delivery vehicle to the target cells and successfully generates an enhanced immune response. In our opinion, these studies pose a strong case for the use of gelatin as a biomaterial to generate novel adjuvants for antitumoral or antiviral vaccines.

4.4 Alginate

Alginate is a naturally available brown algae polysaccharide. It is composed of β -D-mannuronic acid and α -L-guluronic acid [54]. Sodium alginate is easily soluble in aqueous solvents and readily forms hydrogels with multivalent cations like Ca^{2+} , Ba^{2+} , and Fe^{3+} [55]. Numerous studies have reported a variety of techniques to control alginate gelling and its chemical and physical properties for specific applications [56]. Therefore alginate hydrogels are adaptive and versatile natural biomaterials and find a variety of application in the biomedical field [57].

In one particular study, the hypothesis of delivering DCs in an injectable hydrogel matrix was tested to achieve larger time periods at a particular site by harboring DCs and concentrating factors secreted by DCs to attain proinflammatory response [58]. A self-gelling alginate

formulation was developed by mixing calcium loaded alginate microspheres with soluble alginate solution and DCs (Fig. 4.3).

This formulation was observed to rapidly gel in vivo. Upon subcutaneous (s.c.) injection in mice, the alginate microspheres containing activated DCs attracted host DCs and T cells to the injection sites while some of the injected DCs also traveled toward the lymph nodes. Also, recruitment of activated antigen specific T lymphocytes to the alginate matrix was

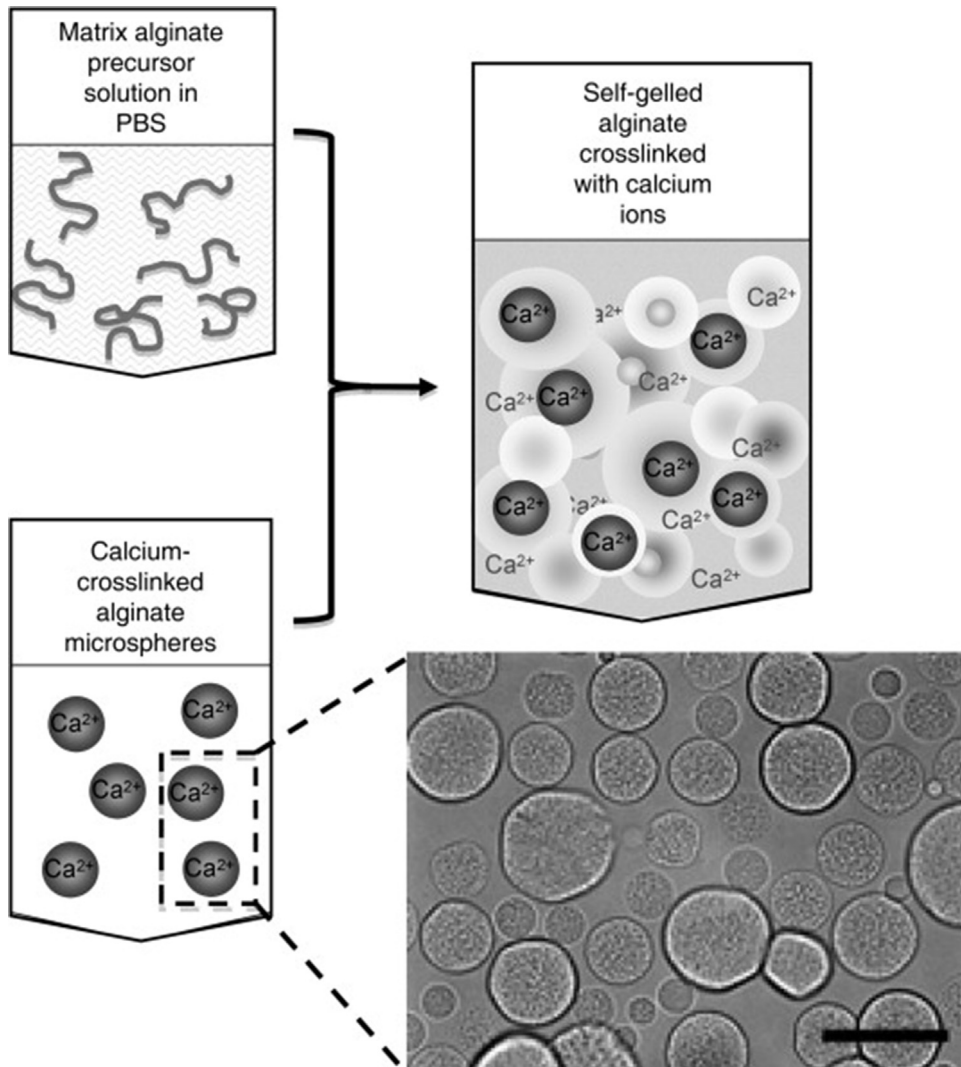


FIGURE 4.3 A schematic of self-gelling calcium-loaded alginate microspheres is shown [58]. Calcium cross-linked alginate microspheres are mixed then with PBS. Upon diffusion of the calcium into the surrounding solution, crosslinking of the soluble alginate is induced due to which a gel is formed. The entire process takes place within minutes. *PBS*, Phosphate buffer saline.

observed which was dependent on the presence of DCs. This DC/alginate system can hence be a platform for immunotherapy to concentrate immune cells at the tumor or injected site in the presence of supporting factors when delivered through alginate microspheres.

Another study investigated the development of an injectable hydrogel system to enrich the local concentration of DCs without the induction of maturation or activation [59]. The group prepared alginate hydrogels to use as physical scaffold for cell infiltration. The alginate hydrogels also control the release of GM-CSF. A sustained release of GM-CSF from the porous alginate gels resulted in the accumulation of cells in the hydrogel. These cells were found to contain high amounts of CD11b⁺CD11c⁺ DCs and further quantification of cell surface markers led to the indication of these DCs being immature. Therefore the study was successfully able to demonstrate the interaction between alginate hydrogels and DCs by the accumulation of immature DCs providing basis for development of polymeric delivery systems for vaccines.

4.5 Chitosan

Chitosan is one of the most abundant natural polymers occurring in nature [60]. It is a linear cationic polysaccharide and is synthesized by deacetylating chitin. Chitosan is widely used because of its biodegradable and biocompatible nature. It is a nontoxic biopolymer that also possesses the attributes of hydrating and antibacterial agents [61]. They can be utilized in various forms, for example, beads [62], nanoparticles [63], nanofibers [64,65], membranes [66], and scaffolds [67,68]. Due to these reasons chitosan is used in biomedical applications like gene and drug delivery, tissue engineering, and wound healing [69].

Reports suggest that for DC-mediated cancer immunotherapy, it is imperative to induce monocyte differentiation into DCs. In one study, monocytes were isolated from mouse bone marrow and were cultured on chitosan substrate instead of the tradition all tissue culture polystyrene (TCPS) to investigate the result of chitosan culture system on induction and tumor protection of DCs [70]. It was observed that when compared to TCPS, chitosan culture system increased monocyte aggregation and detachment. Also, there was an increase in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction activity and expression of DC marker CD11c. It was also observed that chitosan could enhance lipopolysaccharide stimulated SCs to secrete higher amount of interleukin (IL)-12. Moreover, when compared to TCPS, upon vaccination of tumor lysate-pulsed DCs harvested from chitosan culture, an enhancement in cytotoxic T-lymphocyte was observed with an increased antitumor effect. Hence, the study successfully demonstrated that treatment of DCs with tumor lysate following culture on chitosan has future potential DC-based vaccine applications.

In another study, researchers explored the effect of chitosan encapsulation on the uptake, activation and presentation of antigen by DCs [71] and other APCs. Antigen-encapsulated chitosan particles (AgCPs) were created by changing the formulating conditions to obtain varying sizes. Through uptake studies it was observed that the size, concentration, and incubation time of the AgCPs had influence over the uptake by DCs. The maximum uptake of AgCPs was seen post incubation with DCs and macrophages for 24–48 hours. Therefore

an increased upregulation of surface activation markers on DCs was observed because of these AgCPs that enhanced the release of proinflammatory cytokines.

4.6 Synthetic polymer biomaterials

Synthetic biomaterials are a viable alternative to biological polymers like collagen, chitosan, silk, etc. Some of the key advantages of synthetic polymeric biomaterials are (1) low degradation rate, (2) solid mechanical structure, and (3) uniformity of its microstructure. They also possess a longer shelf life and are easily reproducible [72]. Modulation of structural, mechanical, and chemical properties can be achieved by generating polymers or block copolymers [73,74]. Some of the commonly used synthetic polymer biomaterials include poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG), and polyvinyl alcohol (PVA).

4.7 Poly(lactic-co-glycolic acid)

wPLGA is a widely used copolymer known for its inherent property of biodegradability and biocompatibility [75,76]. It has been used in various FDA approved therapeutic devices [77,78]. It degrades in the presence of water through hydrolysis of its ester linkages and has a glass transition temperature in the range of 40°C–60°C. PLGA nanoparticles have shown to possess various properties like enhancing the cellular uptake of DCs, protection from degradation, controlled release of antigen, and elimination of booster-dose [79–81].

One of the major roles of DCs is in priming of CD8⁺ T cells for inducing inflammatory responses [82,83]. One of the emerging treatments for cancer therapy is induction of tumor-specific CD8⁺ T cells using tumor-specific vaccines [84–86]. This treatment is highly dependent on the sufficient delivery of the vaccine components to DCs. A major disadvantage of this treatment is that upon s.c. injections, a high fraction of the vaccine either induces other immune cell activity or gets cleared from the body [87]. In one of the studies, agonistic α CD40-mAb-coated biodegradable PLGA nanoparticles were employed which were encapsulated with the protein antigen, Pam3CSK4 and adjuvant, poly(I:C) [88]. These nanoparticles were specifically targeted toward CD40 to upregulate the CD8⁺ T cell response. Upon s.c. injection, an increased efficacy of selective delivery to DCs was observed leading to better priming of CD8⁺ T cells. This study concluded that the PLGA-based nanoparticle composite was able to get an enhanced T-cell response and can be a promising treatment modality for improved protein cancer vaccine.

In one particular study, PLGA was found to induce DC maturation and assisted in the increase of human monocyte-derived DC adhesion [89]. It was seen that PLGA was able to upregulate the integrin receptor gene expression (measured using reverse Transcription polymerase chain reaction) when compared to TCPS (Fig. 4.4).

Interestingly, after various antibody-blocking techniques it was observed that the adhesion to PLGA as well as TCPS was dependent on β_2 integrin while being independent of β_1 . CD86 expression or level of DC maturation was lowered following the inhibition of β_2 -mediated adhesion through antibody blocking. β_2 integrins were found to be in direct contact with the PLGA surface as well as localized near biomaterial-adherent DC podosomes.

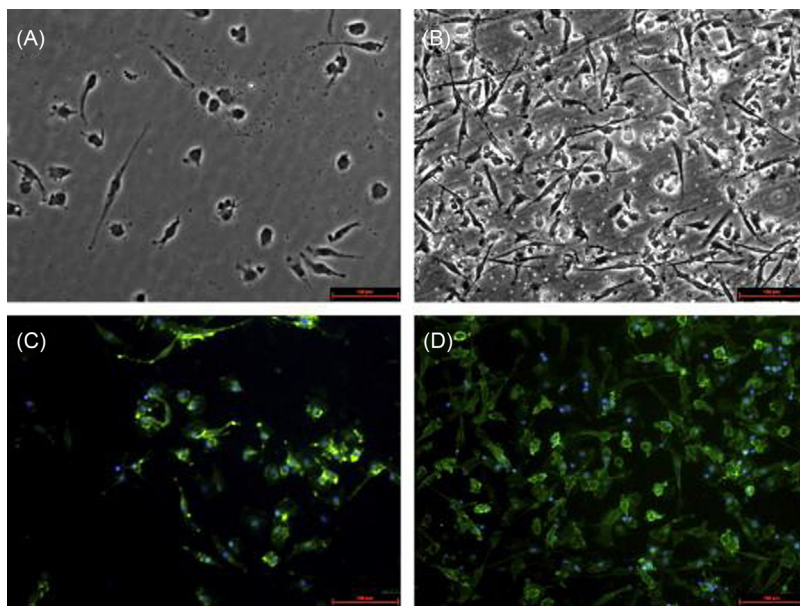


FIGURE 4.4 (A and C) DCs were cultured on plain TCPS and (B and D) PLGA-coated films for 24 h [89]. Cells were stained with DC-SIGN-FITC and DAPI for visualization of nuclei. In the case of DCs which were cultured on TCPS, round morphology was observed while DCs cultured on PLGA were shown to have more cellular adhesion to the surface. DC, Dendritic cell; PLGA, poly(lactic-co-glycolic acid); TCPS, tissue culture polystyrene.

Hence, this study showed that PLGA surface played a role in regulating the state of DC maturation.

Another study investigated the enhanced DC maturation post treatment with tumor lysate antigen-encapsulated PLGA nanoparticles (PLGA-NP) [90]. The efficacy of PLGA-NP-mediated tumor lysate delivery to DCs was assessed by CD3⁺ T-cell stimulation in a mixed lymphocyte culture of T cell with DCs. It was observed that the cumulative release of initial protein content in PLGA-NPs was greater. Also, a significant increase in IL-12 and IFN- γ production was reported because of the T cells simulated with lysate-pulsed DCs.

These studies showed that PLGA-based polymers or nanoparticles are promising vehicles for inducing maturation and stimulation of DCs via protein antigen delivery. PLGA-based composites also help in increased stability of antigen, targeted delivery, and slow release of antigens and enhanced immunogenicity.

4.8 Polyethylene glycol

PEG is a hydrophilic molecule and is a polymerized form of ethylene glycol. Generally, their molecular mass is over 20,000 g/mol but they are also available in a wide range of molecular weight. PEG has been used for a variety of therapeutics and treatments because it is generally considered biologically safe and inert [91,92]. Because of these properties, PEG has been used for various biomedical applications [93–95].

In one study, functionalized PEG hydrogels with IL-10 and transforming growth factor (TGF)- β 1 (immunosuppressive proteins) were utilized to reduce the maturation of DCs [96]. Notably, IL-10 and TGF- β 1, retained bioactivity toward DCs when they are

immobilized on the hydrogel surface. A downregulation of activation markers and reduced capacity to activate T cells were observed for PEG hydrogels interacting with primary bone marrow-derived DCs (BMDCs). When a second signal, which was able to promote cell-material interactions was introduced to promote BMDC-material interactions along with tolerizing signals, it was found that the PEG hydrogels enhanced signaling toward BMDCs. This was also confirmed by a significant reduction in maturation markers. Another study investigated the effect of PEGylation on trafficking and the accumulation of particles in draining lymph nodes (dLNs) [97]. At the single cell level, increased uptake by DCs was observed due to PEGylation. The results also indicated that PEGylation plays a vital role in trafficking of particles through increased internalization by migratory DCs or enhancing trafficking in lymphatic vessels to dLNs. This study gives a comprehensive (to the best of our knowledge) understanding into the use of PEGylated particles to develop novel synthetic vaccines for various diseases.

In another important study, PEG conjugated adjuvants were utilized for immunosuppressive applications. In this study, 1Z1, a composite made of TLR7 ligand and six units of PEG was generated as a novel innate immune response. 1Z1, a composite made of TLR7 ligand and six units of PEG was generated as a novel innate immune modulator [98]. There was a decreased potency of 1Z1 to induce cytokine production by DCs than the parent ligand. This drug highly upregulated programmed death ligand 1 (PD-L1) and IL-1 receptor-associated kinase M (IRAK-M) while only a slight increase in MHC class II, CD80, and CD86 activation markers was observed. The study reported that daily treatment of 1Z1 reduced histologic islet inflammation. It also prevented the clinical onset of hyperglycemia. Daily administration of 1Z1 also induced increased PD-L1 expression in CD11c⁺ population in peripancreatic lymph nodes. Therefore PEGylated biomaterials also play an important role in pharmaceutical modulation of DC modulation and function and can potentially be used for autoimmune disease treatment.

4.9 Blends

Numerous natural and synthetic biomaterials have been employed for various applications in the biomedical field, namely drug delivery, implants, and tissue engineering scaffolds among others [99]. Although, natural biomaterials are biocompatible, biodegradable, and have a higher bioactivity they lack the reproducibility and mechanical properties of synthetic biomaterials [100]. On the other hand, synthetic biomaterials have disadvantages, in that they lack the desired bioactivity and are not biocompatible causing inflammation and other side effects [101]. To solve this problem, researchers designed composite polymeric biomaterials or blends that could have the desired properties of the biomaterials involved needed for specific biomedical applications [102,103].

4.10 Poly(lactic-co-glycolic acid)-chitosan

Plasmid DNA (pDNA) vaccines protect against many diseases [104] including rabies [105] but degrade quickly and lack in their uptake by APCs [106]. To address this issue,

researchers investigated novel PLGA-collagen nanoparticles as a way to deliver vaccines [107]. The cationic PLGA-chitosan nanoparticle blend could attract the negatively charged pDNA onto its surface. The pDNA covered PLGA-chitosan nanoparticles were dispersed in a poloxamer 407 hydrogel for controlled release of pDNA. It was observed that, the uptake of these nanoparticles by the DCs was dependent on time and concentration. Furthermore, when a study was performed wherein the DCs were pulsed with pDNA only in one case and pDNA covered PLGA-chitosan nanoparticle vaccine in the other, DCs showed a higher antigenicity of pDNA covered PLGA-chitosan nanoparticle over pDNA only. This suggests that the PLGA-chitosan nanoparticle blend that could upregulate the activation of DCs, showed an enhanced cell uptake and were stable during the prolonged release of pDNA.

4.10.1 Monomethoxy poly(ethylene glycol)-poly(lactic-co-glycolic acid)

In one of the studies, a novel monomethoxyPEG-*co*-PLGA copolymer (mPEG-PLGA hydrogel) was developed with an aim to produce a cell-based cancer vaccine in situ [108]. A two-step approach was used to achieve the goal (Fig. 4.5).

In the first step, a sustained release of thermosensitive mPEG-PLGA hydrogels was observed from the injected GM-CSF (a known chemoattractant). Due to this, a large amount of DCs were recruited to the site of the injection. In step two, an antigen was introduced to activate the accumulated DCs, and induced antigen presentation by DCs, thus triggering an immune response. This approach was successfully investigated in prophylactic and therapeutic models of murine melanoma, where a high level of tumor-specific immunity was observed

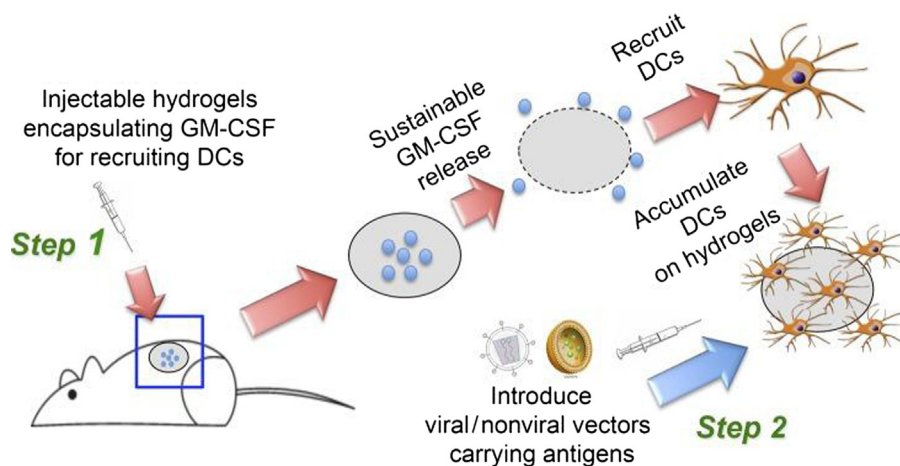


FIGURE 4.5 A schematic representation of a two-step approach for a cancer vaccine is illustrated [108]. Step one includes the controlled release of GM-CSF from mPEG-PLGA composite hydrogels and the administration of host DCs recruited by it to the site. Step two involves the use of immunogens carried by viral or nonviral vectors that can be delivered to the resident DCs in hydrogel in situ to upregulate the antigen uptake efficacy and hence improving immunity against cancer. DC, Dendritic cell; mPEG, monomethoxy poly(ethylene glycol); PLGA, poly(lactic-co-glycolic acid).

[108]. Therefore this composite copolymer hydrogel approach shows high potential as a new approach for in situ modulation of cells for various cell therapies.

4.11 Conclusion and future directions

In conclusion, DCs, provide an effective target for inducing both innate and adaptive immunity, for various disorders including cancer, autoimmune disorders, inflammation, and infections. Targeting DCs has been a very important and successful strategy in inducing both proinflammatory, antiinflammatory, and tolerogenic responses. More importantly, these cells have been targeted both in clinic and in research for generating effective immune responses.

Biomaterials provide a unique opportunity to not only act as delivery vehicles to target extracellular receptors (e.g., integrins) but at the same can be used to target intracellular receptors of these cells (e.g., toll-like receptors, STING pathway). This ability of modulating multiple pathways of DCs simultaneously, by drug delivery or providing a substrate for adhesion/growth, make biomaterials uniquely qualified to make a big impact on the field of immunotherapies.

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Impact of biomaterials' physical properties on cellular and molecular responses

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Abbreviations

ECM	extracellular matrix
EGF	epidermal growth factor
FBGCs	foreign body giant cells
FBR	foreign body reaction
FGF	fibroblast growth factor
GM-CSF	granulocyte macrophage colony stimulating factor
IFN- γ	interferon- γ
IL-10	interleukin-10
IL-1 β	interleukin-1 β
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinases
PDGF	platelet-derived growth factor
PEG-PC	poly(ethylene glycol) dimethacrylate and 2-methacryloyloxyethyl phosphorylcholine
PLGA	poly(lactic-co-glycolic acid)
TGF- β	transforming growth factor- β
TNF- α	tumor necrosis factor- α
VEGF	vascular endothelial growth factor

5.1 Introduction

Exogenous biomaterials have been widely explored in medical implants/devices for decades, including artificial organs, heart valves, biosensors, catheters, and scaffolds for

tissue engineering as well as in drug delivery systems. However, it has become apparent during the past several decades that some important challenges must be tackled and overcome. Among these, biocompatibility and long-term continuous function are problematic for the clinical success of many medical implants and devices. For example, continuous glucose monitoring with the assistance of an implantable glucose sensor is a major goal in the treatment of diabetes to avoid multiple finger-pricks. However, most of the commercially available implantable glucose sensors have a limited life span (days to weeks) and this is a result of dysfunction due to the foreign body reaction (FBR), which is triggered by tissue trauma during implantation as well as by tissue perturbation as a consequence of the persistent presence of the implanted sensor.

Post implantation, a series of cellular responses, along with molecular responses, are initiated, including acute inflammation, chronic inflammation, wound healing, and finally fibrous encapsulation. These host responses to the implanted materials determine the longevity of the medical implants and devices. It is important to note that the extent and duration of the cellular and molecular response depends on many factors, such as the site of implantation, the extent of injury, as well as the chemical and physical properties of the biomaterials (Fig. 5.1).

In this chapter, an overview of the basic mechanisms of the cellular and molecular events to biomaterials, as well as the effects of material physical properties on the host response, is discussed.

5.2 Cellular and molecular response following implantation

An implant/device is generally placed into patients by insertion, injection, or surgery, causing injury to vascularized connective tissue. A series of inflammatory events is then initiated, as a means of replacing damaged cells and tissues to heal and reconstruct the injured implantation site. The cells/biomolecules of interest in each inflammatory event, as well as their secreted chemical mediators are listed in Table 5.1.

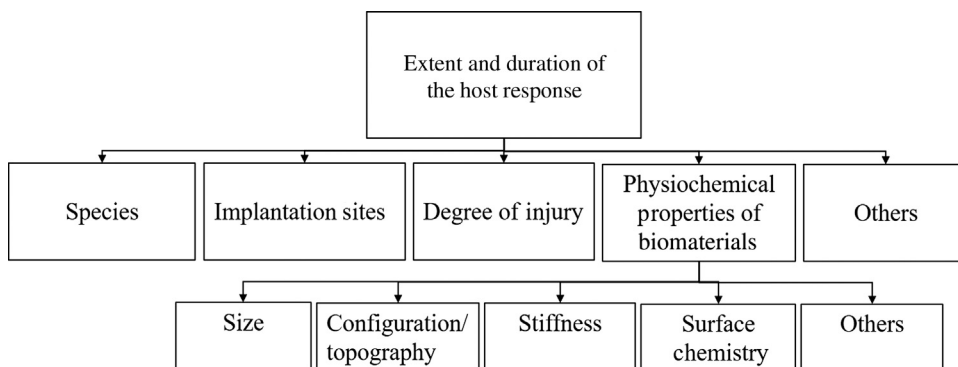


FIGURE 5.1 Factors that impact on the extent and duration of the host cellular and molecular response.

TABLE 5.1 A list of the sequence of inflammatory events and the relevant cells/molecules.

Inflammatory events	Cells/biomolecules of interest	Secreted chemical mediators
Blood materials interaction	Fibrinogen, fibronectin, albumin, complement, and immunoglobulin	Plasma proteases
Acute inflammation	Neutrophils: phagocytosis, ECM degradation, and cell recruitment	MMP, MIP, IL-8, reactive oxygen species
	Mast cells: cell recruitment	Histamine
Chronic inflammation	Macrophages: phagocytosis, antigen presentation, tissue repair, and remodeling	IL-6, IL-8, IL-10, TNF- α , PDGF, GM-CSF, IFN- γ
	Lymphocytes: macrophage activation and foreign body giant cell formation	IL-4, IL-10, IL-13
Wound healing	Fibroblasts: granulation tissue formation	FGF, PDGF, EGF, TGF- β
	Endothelial cells: angiogenesis	VEGF
Foreign body reaction	Foreign body giant cells: breakdown of foreign materials and phagocytosis	MMP, oxygen and nitrogen species
Fibrous capsule formation	Myofibroblasts: wound contraction, tissue remodeling	TGF- β , reactive oxygen species
	Collagen: fibrosis	

ECM, Extracellular matrix; *EGF*, epidermal growth factor; *FGF*, fibroblast growth factor; *GM-CSF*, granulocyte macrophage colony stimulating factor; *IFN- γ* , interferon- γ ; *IL-10*, interleukin-10; *IL-13*, interleukin-13; *IL-4*, interleukin-4; *IL-6*, interleukin-6; *IL-8*, interleukin-8; *MIP*, macrophage inflammatory protein; *MMP*, matrix metalloproteinases; *PDGF*, platelet-derived growth factor; *TGF- β* , transforming growth factor- β ; *TNF- α* , tumor necrosis factor- α ; *VEGF*, vascular endothelial growth factor.

5.2.1 Blood-materials interaction

Immediately following implantation, proteins migrate from the blood and body fluids to the implantation site. Protein adsorption occurs within nanoseconds, forming a protein layer on the surface of the biomaterials and becomes a mediator between the biomaterials and the cell layers. The well-known Vroman effect describes the dynamic processes of protein adsorption and exchange, in which highly concentrated and high mobility proteins deposit on the biomaterial surface first and are finally replaced by less mobile proteins that have higher surface affinity [1].

Protein types, concentration, conformation and/or orientation, and the biomaterial surface chemistry govern cell recognition and other downstream cascades of body responses. Important proteins involved include: (1) albumin, which has the highest blood concentration of approximately 40 mg/mL, and can bind with various hydrophobic ligands including fatty acids, steroids, and anesthetics [2,3]. Due to its smaller size and higher concentration compared to other plasma proteins, albumin is reported as the first protein that reaches a biomaterial surface following implantation, and thus albumin deposition may make a contribution to the initial events of cell recognition, although it is a nonadhesive serum protein. (2) Fibrinogen is the third primary plasma component with a concentration of 2–4 mg/mL in blood [4]. Biomaterials exposed to blood or body fluids become enriched in fibrinogen. Fibrinogen undergoes denaturation and

exposes integrin ligands following adsorption, allowing integrin-mediated leukocyte adhesion to biomaterials. The extent of fibrinogen deformation on an implanted biomaterial surface has a strong correlation with the degree of acute inflammatory response. It is also reported that fibrinogen plays an important role in the initiation of platelet adhesion. (3) Fibronectin and vitronectin are important components of extracellular matrix (ECM). These two glycoproteins are not only important for cell adhesion, but are also essential for regulation of the inflammatory response. The conformational change of fibronectin impacts cell migration, proliferation, and adhesion as well as the release of chemical mediators to assist the wound healing process [5,6]. (4) Other protein molecules, such as complement, and immunoglobulin also deposit on biomaterial surfaces and activate different cascade systems to mediate blood coagulation/thrombosis or other inflammatory events.

During the very early process of blood and material interaction (within minutes to hours post implantation), provisional matrix formation occurs along with protein adsorption. The provisional matrix is mainly composed of fibrin and provides a support for deposited proteins, recruited cells and a number of cytokines, chemokines, and growth factors, which facilitate the subsequent cellular response and wound healing processes.

5.2.2 Acute inflammation

Following the initial blood and material interactions, acute inflammation is initiated by the release of chemical mediators from the provisional matrix and neutrophils are recruited. Activated neutrophils work as the first defender, degrading or phagocytosing microorganisms, damaged cells, and foreign bodies. They also produce and secrete various chemical mediators, in order to break down the components of the ECM [e.g., matrix metalloproteinases (MMP)], to kill pathogens (e.g., reactive oxygen species), or to recruit other inflammatory cells [e.g., interleukin-8 (IL-8), macrophage inflammatory protein (MIP)] [7,8]. The amount of neutrophils at the implant site is dependent on the extent of injury as well as the surface chemistry of the biomaterials. The degree of neutrophil infiltration may have an effect on later fibrotic responses [9]. Most commonly, neutrophils have a short lifetime, and are prevalent at biomaterial surfaces for about 1–2 days. However, there is also evidence showing that the long-term recruitment of neutrophils indicates chronic-active inflammation, due to either infection or persistent perturbation of tissues by implants [10]. During the acute inflammatory response, mast cells also modulate the recruitment of phagocytes by the release of histamine. Acute inflammation usually has a quick resolution (less than 1 week).

5.2.3 Chronic inflammation

Cytokines/chemokines produced by neutrophils, mast cells, platelets, and clots give rise to the infiltration of mononuclear cells, including monocytes, macrophages, and lymphocytes. The prevalence of these mononuclear cells at the implantation site is considered chronic inflammation, and is usually caused by toxic leachable components from biomaterials or by infection [11,12]. The FBR usually consists of macrophages and foreign body giant cells (FBGCs) with the development of granulation tissue. During this phase, increased levels of chemo-attractants secreted from neutrophils suppress the recruitment of neutrophils and they

start to undergo apoptosis. Macrophages arriving at the damaged tissue sites clear the neutrophils and other cell debris. The infiltrated macrophages further secrete chemo-attractants, including platelet-derived growth factor (PDGF), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and granulocyte macrophage colony stimulating factor (GM-CSF) to recruit more macrophages to implantation site [13–15]. In response to different inflammatory stimuli, macrophages undergo polarization and display two phenotypes: pro-inflammatory macrophages (M1 macrophages) and antiinflammatory macrophages (M2 macrophages). M1 macrophages respond to interferon- γ (IFN- γ), degrading and clearing debris and foreign materials, whereas M2 macrophages are in response to interleukin-4 (IL-4) or interleukin-10 (IL-10), promoting angiogenesis as well as tissue repair and remodeling (Fig. 5.2). However, macrophages have high plasticity and their phenotypes are reversible to some extent based on certain environmental stimuli. For example, the removal of IL-10 was able to alter the phenotype of macrophages from M2 to M1 [16]. Failure of clearance of M1 cells by M2 cells may lead to the release of cytotoxic components from M1 cells and thus contribute to further infiltration of inflammatory cells, while M2 cells are known to trigger giant cell formation and thus foster fibrosis [15,17,18]. Therefore, the balance between M1 and M2 cells plays an important role in chronic inflammation.

5.2.4 Wound healing

The healing response takes place at the injury site within 24 hours post implantation. Upon adhesion of monocytes/macrophages, activated macrophages produce various growth factors, which are vital to the recruitment of fibroblasts and the formation of new blood vessels. These growth factors include fibroblast growth factor (FGF), PDGF, epidermal growth factor (EGF), and transforming growth factor- β (TGF- β). From a histologic perspective, granulation tissue formation involving the proliferation of fibroblasts and endothelial cells at the implantation site is the hallmark feature of the wound healing process. Angiogenesis is vital for wound

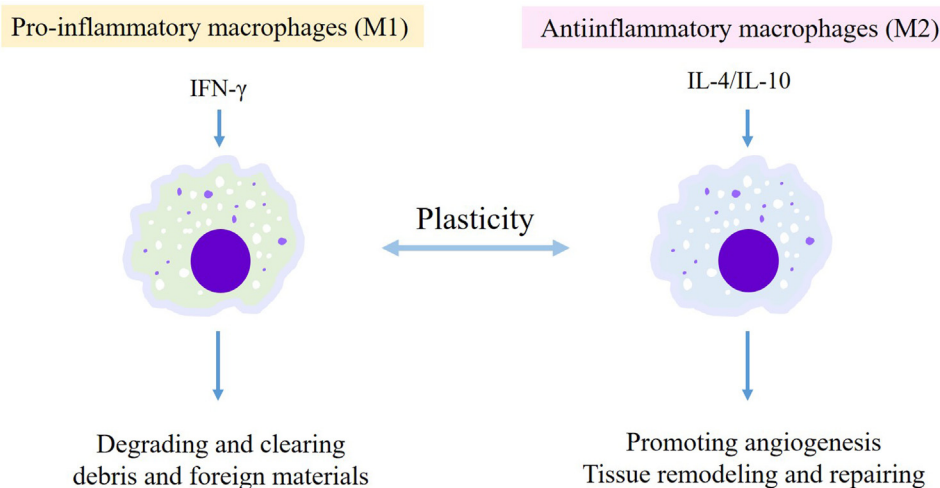


FIGURE 5.2 Polarization of macrophages.

healing as well as for the long-term maintenance of some implantable devices such as glucose sensors. A well-vascularized tissue environment can improve the residence time of glucose sensors. Vascular endothelial growth factor (VEGF) has been widely investigated and it has been shown that the codelivery of VEGF and dexamethasone using poly(lactic-co-glycolic acid) (PLGA) microsphere/polyvinyl alcohol hydrogel composite coatings can mitigate both acute and chronic inflammation and consequently improve biocompatibility and ensure in vivo performance of biosensors [19,20]. Granulation tissue consists of a loose network of type III collagen, ECM proteins, capillary sprouts, macrophages, and fibroblasts. Fibroblasts are mainly derived from primary mesenchymal cells, but can also stem from epithelial cells through epithelial–mesenchymal transition, and from circulating monocyte-derived fibrocytes [21,22]. Myofibroblasts are one of the phenotypes of fibroblasts that show the characteristics of smooth muscle cells, helping wound contraction.

5.2.5 Foreign body reaction

FBGCs are known as the hallmark of the FBR and are formed by the fusion of macrophages. The fusion of macrophages is induced by interleukin-13 (IL-13) and IL-4. Macrophages are able to engulf and digest foreign materials with the longest dimension of less than 5–10 μm . When the material is too large for macrophages to phagocytose, multiple single macrophages fuse together and form FBGCs in an attempt to phagocytose and degrade foreign materials by secreting MMP [23]. When the foreign body is too large (over 50–100 μm), “frustrated phagocytosis” takes place at the implantation site, where macrophages/FBGCs release various chemical mediators, such as oxygen and nitrogen species, proteases, and acid to degrade the foreign material [24,25]. The mechanisms regarding macrophage fusion and FBGCs formation have not been well-studied. However, it has been reported that FBGC formation needs appropriate environmental stimuli as well as certain adsorbed protein layers on the biomaterial surface. FBGCs are multinucleated cells and could be present surrounding implants for their entire lifetime.

5.2.6 Fibrous capsule formation

In general, the formation of FBGCs and chronic-active inflammation may ultimately lead to fibrous encapsulation around the implant, which is considered as the last phase of the wound healing process to the presence of biomaterials. Fibrous capsule formation is a mechanism by the host to isolate the implant from the rest of the body. Injured cells are replaced by either parenchymal cells of the same type to remodel and reconstitute normal tissue/organ structure, or connective tissue which is mainly composed of collagen fibers and fibroblasts. As mentioned previously, granulation tissue formation is initiated at the beginning of the healing response. During the maturation of granulation tissues, the major component, type III collagen, is gradually replaced with type I collagen. A thin collagenous capsule surrounding biomaterials with a relatively quiescent reaction is acceptable for many types of implants. However, for biosensors, this is not acceptable as it can minimize or eliminate analyte access to the biosensor, rendering the biosensor ineffective.

The timing of the entire inflammatory process including fibrotic encapsulation has been shown to be species dependent. Recent studies have reported that fibroblast recruitment and collagen deposition starts earlier in mini pigs compared to rats [26,27]. The variation in the onset of fibrous encapsulation among different species is an important indicator of differences in the development and progression of chronic inflammation. Consequently, the release pattern of antiinflammatory drugs to overcome the inflammatory reaction through application of an antiinflammatory delivery system should be species specific.

5.3 Impact of physical properties on modulation of the host response

Physicochemical properties of implants, including size, topography, stiffness as well as surface chemistry, have significant impact on biocompatibility. Tuning these factors is likely to suppress protein adsorption, limit the release of chemical mediators and change the cell infiltration profile, thus further contributing by preventing/reducing the FBR and immunogenicity. Translation of implantable biomaterials and medical devices requires a thorough understanding of the effects of material physiochemical properties on their safety profile.

5.3.1 Size

The interaction between cells and particles varies at nanoscale (<1000 nm), microscale (>1 μm), and macroscale (>1 mm) (Table 5.2). Particles in the nanosize range are easier to be phagocytosed and may cause cytotoxicity, whereas the FBR becomes more dominant in larger-sized objects. Phagocytes with a diameter ranging from several micrometers to 30 μm , such as neutrophils, macrophages, and mast cells, are able to engulf particles smaller than 10 μm . Nanoparticles are readily taken up by phagocytes and the accumulation of nanoparticles may result in reduced functional activity of phagocytes and may also trigger the secretion of inflammatory cytokines, such as IL-1, IL-6, and TNF- α [28,29]. The magnitude of the inflammatory response caused by nanoparticles also correlates with their size [25,30]. For example, smaller sized titanium particles (20 nm in diameter) showed a chronic-activated higher inflammatory response compared to larger size particle (250 nm in diameter) in the lungs of rats [31]. The underlying mechanism of this phenomenon may be that smaller sized particles have a larger exposed surface area available to react with the cells and inflammatory

TABLE 5.2 The impact of object size on cellular response.

Size of objects	Phagocytosis	Cells
Nanoscale < 1000 nm	Easy to be phagocytosed May cause toxicity	Neutrophils, mast cell, macrophages
Microscale 1 μm to 1 mm	< 10 μm : eliciting phagocytosis Up to 100 μm : FBGCs formation	Macrophages, FBGCs
Macroscale > 1 mm	Unlikely to be phagocytosed but the amount of enzyme released by phagocytes is associated with the dimension of implants	Macrophages, FBGCs

FBGCs, Foreign body giant cells.

mediators. However, it does not always follow that smaller sized particles trigger more severe inflammatory activities. A conflicting *in vitro* study demonstrated that 40 nm gold nanoparticles stimulated the secretion of cytokines (TNF- α , IL-12, IL-6, and GM-CSF), while 20 nm gold particles triggered no inflammatory reaction in dendritic cells [32]. The degree of cellular and molecular responses to nanoparticles is due in part to particle size. Other factors including surface charge and shape also influence the inflammatory response, which may explain why the size effect appears to be context dependent.

Micro-sized particles have received increasing attention due to their controllable drug release profiles. Small microparticles ($<10\text{ }\mu\text{m}$) can elicit phagocytosis, while microparticles with a larger size can trigger the formation of FBGCs, which are able to engulf large sized particles (up to $100\text{ }\mu\text{m}$). There are a few studies addressing the relationship between microparticle size and the host inflammatory response. PLGA-based microspheres are widely used as vehicles for both local and systemic drug delivery. A study on the extent of FBR toward implants consisting of different sized PLGA microspheres demonstrated that size matters in the different stages of the inflammatory reaction [33]. Phagocytosis occurs at the implantation site of small-sized PLGA microspheres ($5\text{ }\mu\text{m}$), whereas no phagocytosis was observed at the implantation site of large sized PLGA microspheres ($30\text{ }\mu\text{m}$). The $5\text{ }\mu\text{m}$ PLGA microspheres recruit more macrophages than the $30\text{ }\mu\text{m}$ PLGA microspheres. In addition, compared to the $30\text{ }\mu\text{m}$ PLGA microspheres, higher collagen deposition appeared at the implant site of $5\text{ }\mu\text{m}$ PLGA microspheres. Investigation of the size effect of alginate spheres on the innate immune response demonstrated that larger-sized spheres are more biocompatible than smaller sized spheres.

Macroscale objects are unlikely to be phagocytosed. Inflammatory cells still deposit on the surface of biomaterials and release more enzymes to degrade materials. The larger the dimensions of the implant, the greater the amount of enzyme release. Implant size was also reported to change collagen deposition. Implantable glucose sensors for continuous glucose monitoring are plagued by biofouling and negative inflammatory reactions at the implantation site, resulting in fibrous encapsulation around the sensor and ultimately significantly shorten the effective lifetime of implantable glucose. The extent of FBR at the implantation site has been attributed to the dimensions of implantable sensor size. Large ($0.75 \times 0.75 \times 9\text{ mm}^3$), medium ($0.5 \times 0.5 \times 5\text{ mm}^3$), and small ($0.3 \times 0.3 \times 3\text{ mm}^3$) sized dummy sensors were placed in rats over different durations of implantation [34]. It was shown that sensor dimensions did not significantly influence acute inflammation. The thickness of the fibrous capsule, however, increased with the sensor dimensions, indicating that sensor size plays an important role in chronic inflammation. On the other hand, acute inflammation was shown to be dependent on the size of the needle used for implantation of the biosensor device, the larger the needle the greater the acute inflammatory response.

5.3.2 Configuration and topography

The role of topography and configuration of implants on the cellular and molecular response to the host has also been widely explored. At the nanoscale or microscale, particles are readily phagocytosed. The phagocytosis of particles by macrophages has also been attributed to the shape of particles [35]. Compared to spheres and oblate ellipsoids, prolate ellipsoids exhibited the lowest internalization. Macrophage phagocytosis is an actin-involved

remodeling process, in which curved particles with a large aspect ratio require a greater degree of actin remodeling for internalization. This properly explains that prolate ellipsoids showing larger aspect ratios have more difficulty in activating the uptake by macrophages. Studies on worm-like micellar particles also showed evidence that particles with higher aspect ratios can more easily escape phagocytosis [36].

At the macroscale, it has also been shown that implants with sharp edges and angles triggered more tissue response than implants with rounded shapes. Three different shapes of polymeric implants were utilized to demonstrate the significance of implant configuration on tissue response [37]. Circular-, triangular- and pentagonal-shaped rods were implanted into the gluteal muscles of rats. Fourteen days post implantation, triangular-shaped implants exhibited the highest cellular and enzyme activity while circular-shaped rods showed the lowest inflammatory reactions, indicating that implants with smooth surfaces caused less irritation and stress to the surrounding tissues than those with sharp edges. Additionally, the microscale and submicron scale topography cue of biomaterials affected cell behaviors. For example, topography-induced variations in macrophage adhesion and infusion as well as cytokine release levels were investigated using parallel gratings with a line width ranging from 250 nm to 2 μ m imprinted on different biomedical polymers [38]. The 2 μ m gratings showed a reduced level of macrophage adhesion and FBGCs fusion compared to planar surfaces. The production of cytokines including TNF- α (pro-inflammatory cytokine) and VEGF (promoting angiogenesis and wound healing) was decreased on the larger grating sizes. These observations were identical on three different materials poly(ϵ -caprolactone), poly(lactic acid), and poly(dimethyl siloxane), showing the independence to surface chemistry to the inflammatory response. The impact of microscale and submicron scale topography on cellular response has also been explored in many other cell types, such as fibroblasts [39], epithelial cells [40], and mesenchymal stem cells [41].

The geometry and topography of scaffolds are also important for tissue engineering. The curvature of the scaffold surface has been shown to result in differences in both cell attachment and migration [42,43]. It has been reported that concave surfaces favor tissue growth and angiogenesis compared to flat and convex surfaces [42]. In addition, the pore structures of scaffolds are also important in the inflammatory response by affecting macrophage behavior and cytokine secretions [44,45]. In general, porous material triggered a lesser extent of FBR (e.g., thinner fibrous capsules) and promoted wound healing [13,46,47]. Studies on subcutaneously implanted poly-hydroxyl-ethyl-methacrylate hydrogel scaffolds showed that porous materials exhibited an enhanced vascular density and a reduced level of fibrosis [47]. It was also reported that the cellular responses are dependent on the size of the pores. M1 and M2 cells showed a different pattern of distribution according to surface porosity. M1 cells (pro-inflammatory cells) were mainly located in the implant porous structure, while the M2 cells (antiinflammatory cells) became dominant immediately outside the implant pores. Even though some other studies have reported that M1 cells are not responsible for fibrosis in the wound healing process, it appears that the pro-healing process upregulates the makers of both M1 and M2 cells. This is understandable, as macrophages are known as plastic cells and the transition between M1 and M2 is related to environmental stimuli. Current studies distinguish M1 and M2 cells based on a single or very narrow range of markers, which may not be able to determine the real functionality of the polarized cells. To further understand the role of macrophages

and their polarization in improving healing, functionality analysis including cytokine expression and release of other chemical mediators is of significance.

5.3.3 Stiffness

Biomaterial stiffness may apply different extents of stress to the surrounding tissues and thus cause varied cellular and tissue responses. As brain tissue is one of the softest tissues in the body, the mismatch of mechanical properties between brain tissues and neural implant materials may be problematic. Thereby, the stiffness of biomaterials has been widely investigated for neural implants. Polyacrylamide substrates of different compliance were used to examine the morphological and inflammatory responses to different mechanical cues [48]. One was close to the stiffness of brain tissues (shear storage moduli $G' = 100$ Pa), while the other one was two orders of magnitude stiffer ($G' = 10$ or 30 kPa). Microglial cells or astrocytes exhibited a rounded morphology or a physiological shape as founded in vivo in soft materials. However, the cells became more elongated and spread-out when in contact with stiff materials. Upregulation of inflammatory genes and proteins mediated in cell adhesion, migration, and activation have been observed in stiff implant materials in cell models. The composites were implants in rat brains for 1 or 3 weeks. Stronger FBR was observed around the stiff part of the implant site at both time points. Furthermore, interleukin-1 β (IL-1 β) proteins, known as pro-inflammatory mediators, were significantly upregulated at the stiff portion of the implanted materials 3 weeks post implantation. This suggests that softer materials that match the stiffness of the surrounding tissues can mitigate FBR. Applying coating materials that are soft may also help.

The mechanical properties and the capability of incorporating drug molecules into the matrix makes hydrogels a popular material in the application of implant coating and tissue engineering. By the modulating stiffness of the hydrogel, protein adsorption and inflammatory cell infiltration can be limited and as a consequence the FBR is further reduced. A panel of poly (ethylene glycol) dimethacrylate and 2-methacryloyloxyethyl phosphorylcholine (PEG-PC) hydrogels with various levels of stiffness showed that stiffness is one of the driving forces of the FBR [49]. Increasing the stiffness of PEG-PC hydrogels increased the fibrous capsule thickness when implanted subcutaneously. Macrophages had more adhesions on stiffer hydrogel and also released more TNF- α cytokines compared to softer materials, indicating stiff materials may be attractive to pro-inflammatory macrophages [49,50]. Interestingly, the total amount of adsorbed protein on softer hydrogels is more than on stiffer hydrogels. However, when it narrows to the specific protein types (related to ECM formation and immune response), a higher percentage of proteins was deposited on the stiffer hydrogels, indicating these specific proteins may be major contributors to macrophage infiltration and ECM remodeling. This study also demonstrated that not only the biomechanics but also the surface chemistry can influence protein adsorption.

However, it is not always true that the softer the material is, the better the biocompatibility of the implant. Orthopedic implants require that the biomaterials be mechanically stable to provide sufficient mechanical support and at the same time can be well tolerated by the body over the entire duration of implantation. Therefore, the biomaterial must have sufficient strength compatible to the stiffness of the surrounding bone tissues. However, the

stability of orthopedic implants is still problematic in clinical applications. Incompatible mechanical stress may cause tissue degradation and trigger pro-inflammatory cytokines release [51,52]. For example, highly expressed IL-1 β and TNF- α can make the microenvironment hostile for bioengineered orthopedic implants, which can disturb cell metabolism and even healthy tissue as well as implant damage. Hence, lowering the local concentration of IL-1 β and TNF- α may be a solution to this problem. It has also been noted that many biomaterials with good stiffness have demonstrated potential in vitro. However, these materials did not favor a highly porous structure and therefore these materials do not possess sufficient ability for cell recruitment and vascularization. Accordingly, a balance between mechanical properties and topography of the materials is necessary to achieve the final success of the applications of orthopedic implants.

5.3.4 Surface chemistry

Surface chemistry also plays a vital role in material biocompatibility. The material surface properties can influence protein adsorption and further interfere with interactions between immune cells and biomaterials. Hence, surface properties, including hydrophilic and hydrophobic characteristics, surface charge, and surface functionality, have been extensively investigated to understand the impact of surface chemistry on protein-surface interaction, as well as cellular and tissue response [53,54].

In general, hydrophobic surfaces favor protein adsorption and conformational change. This is because water molecules are “structured” on hydrophobic surfaces, which gives rise to a low system entropy. When a protein interacts with a hydrophobic surface, it undergoes unfolding, exposing hydrophobic moieties in order to bind to the surface. This process releases “structured” water molecules back to the bulk water and thus the entropy of the entire system increases. The entropy increase through hydrophobic interaction has been widely accepted as the driving force for protein adsorption in most cases [55,56] (Fig. 5.3). Actively adsorbed proteins on a hydrophobic surface trigger cell adhesions as well as the subsequent local immune response surrounding the implants. For example, the adsorption of albumin, fibrinogen, and human factor XII (which are important proteins in blood and materials interaction) was investigated on various modified polyethylene surfaces with different wettability. It was reported that all three proteins had a stronger adherence on hydrophobic surfaces (poorly wettable) than on hydrophilic surfaces

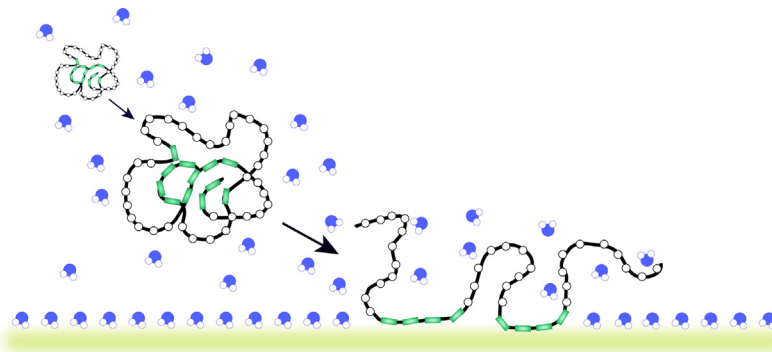


FIGURE 5.3 Entropy-driven protein adsorption.

(highly wettable). Increased adhesion force was observed with contact time, indicating that the proteins undergo surface-induced conformational change during adsorption [57].

Modification of biomaterial surfaces via immobilizing water-loving polymers onto the surface is one potential strategy to improve the biocompatibility of implants. Polyethylene glycol (PEG), for instance, has been widely explored and has shown the ability to resist protein adsorption as well as cell attachment [58–60]. The antifouling capacity of PEG is a result of strong surface hydration and steric expulsion. PEG coatings provide a highly hydrated layer and the strongly bounded water molecules are hard to be replaced by proteins or other biomolecules. Furthermore, when a protein molecule approaches the PEG layer, a repulsive force is developed due to compression of PEG chains and the loss of conformational freedom. It has been reported that the reduction in fibrinogen, IgG, and albumin adsorption onto PEG-coated silicon surfaces, compared to uncoated surfaces was over 60% *in vitro*. In addition, the adhesion and proliferation of fibroblasts also decreased [60]. Silicone rubber is also known as an inert biomaterial and has been widely explored in the application of implants, such as tissue fillers, catheters for drug delivery, and dialysis tubes. However, owing to its highly hydrophobic nature, silicone rubber has shown biofouling problems in long-term use with the accumulation of inflammatory cells and the formation of fibrous capsules surrounding the materials. Therefore, various types of hydrophilic coatings have been investigated to improve the wettability of the surface, including PEO/PEG-based coatings, polyzwitterion-based coatings (copolymers of SBMA and acrylic acid), saccharide-based coatings (carboxymethyl cellulose), and amide-containing-hydrophilic-polymer based coatings (polyacrylamide) [61].

In addition to the hydrophilic and hydrophobic properties of the surface, protein/cell adhesion patterns vary with surfaces bearing different functional groups and charge characteristics. Most serum proteins and cells display negative charges on the surface, which renders positively charged biomaterial surfaces easy to interact with these serum proteins and cells via electrostatic interaction. Amine functional groups are generally positively charged and possess high affinity with serum proteins. It was reported that surfaces rich in amine groups favor the acute inflammatory immune response [62]. Denatured serum proteins, such as fibrinogens and fibronectins trigger cells infiltration (including neutrophils and monocytes) on amine group rich surfaces, as well as strong fibrotic reactions [54,63]. Carboxyl functional groups are ionized to anions at physiologic pH. Even though it has been reported that surfaces modified with carboxyl groups could enhance fibronectin adsorption and cell growth [64], other studies have also reported that the extent of cell growth on surfaces coated with carboxyl groups is dependent on the density of carboxyl groups [65]. Furthermore, the adsorbed fibronectin is easy to be eluted on surfaces rich in carboxyl groups. Under the conditions of similar wettability, surfaces with carboxyl groups showed a relatively minimal immune response and fibrotic reaction compared to other surfaces bearing other functional groups (such as hydroxyl, amine, and amide groups) [54,63,66]. Hydroxyl functional groups show neutral charges. Self-assembled monolayers of alkanethiols on gold have been used to investigate the effect of different terminal functional groups (carboxyl groups, hydroxyl groups, and methyl groups) on cell infiltration and fibrosis formation [67]. With regard to cell recruitment, both methyl and hydroxyl-covered surfaces exhibited high numbers of adhered inflammatory cells, while carboxyl-coated surfaces recruited less inflammatory cells. However, hydroxyl-coated surfaces displayed a thinner fibrotic encapsulation than methyl-modified surfaces, to a similar extent as carboxyl-coated

surfaces. Interestingly, the thickness of fibrous capsules was not associated with the degree of cell infiltration. Hence, the formation of fibrous encapsulation is not only dependent on one factor, such as cell recruitment, rather it is a complicated process. Apart from surface modification by a single functional group, surfaces with mixed functionality have also been determined in terms of protein and cell adhesion. Surface neutrality was known to elicit reduced platelet adhesion and inflammatory response [68]. Surfaces grafted with an equal amount of amine groups and carboxyl groups showed the lowest platelet adhesion [69].

From the perspective of tissue engineering, research focuses on promoting cell adhesion while attenuating host inflammatory response to the scaffold materials. Currently, ECM-derived material is being used for tissue engineering, due to their ability to enhance cell adhesion, proliferation, and differentiation. Collagen, the most abundant protein in the body, is one of the ECM biomaterials applied in bone and eye implants, sponges for burns, etc. However, there is evidence showing that the high crosslink density of these collagen materials with prolonged degradation rate could lead to severe host inflammatory response with the existence of FBGCs, and finally resulted in fibrous encapsulation formation surrounding the implants [70,71]. One of the strategies to reduce FBR is to modulate macrophage polarization to switch from M1 to M2 phenotype, in order to help the resolution of inflammation. It has been reported that chondroitin sulfate functionalized collagen material was able to tune the phenotype of bone-marrow-derived macrophages to the phenotype of antiinflammatory macrophages *in vitro*. Additionally, the *in vivo* data was consistent with *in vitro* findings. Compared to normal collagen, the chondroitin sulfate functionalized collagen material elicited a lower expression level of pro-inflammatory genes [72].

Despite the promising results of improving biocompatibility through surface modification *in vitro/in vivo* correlations may not be achieved, and/or in an acute inflammation *in vivo* model, many reports have lacked long-term implantation data or have failed to show the effects of surface chemistry on the chronic immune response. Accordingly, approach to maximize the effect of surface functionality is still an interesting topic.

5.4 Conclusion

With the broad application of biomaterials and implants, it is necessary to improve the biocompatibility of biomaterials to achieve long-term functionality in a safe manner. Hence, understanding the cellular and molecular response to foreign materials as well as the effect of material properties on the host response becomes crucial in the development and application of biomaterials and implants. Serum proteins (fibronectin, fibrinogen, immunoglobulin, etc.), inflammatory cells (granulocytes, macrophages, fibroblasts, etc.), and chemical mediators (cytokines, chemokines, growth factors, etc.) have been extensively investigated *in vitro* and *in vivo*. However, studies generally focus on single cell type and the corresponding molecules. Owing to the complexity of the *in vivo* environment, the host response to a foreign implanted object is not solely affected by one or two factors. In some cases, *in vitro* studies are not able to correlate with the *in vivo* work. In addition, lacking an appropriate *in vivo* model in the long run limits the investigation of the effect of the chronic inflammatory response to implants. Thereby, the development of an animal model for a long duration of

implantation is needed to provide a better understanding of the host response to biomaterials in a complex in vivo environment.

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Impact of biomaterial mechanics on cellular and molecular responses

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6.1 Introduction

Biomaterials are substances that are tailored for utilization in therapeutic or diagnostic procedures, and are comprised of a broad range of compounds that widely differ in function and structural features. They can be engineered either from naturally occurring biological macromolecules or fully synthetic coatings and have gained significance in varied biomedical fields such as tissue engineering, medical implants, drug delivery, and immunotherapies [1–5]. This wide applicative potential depends on the ability of these materials to provide biocompatible supports (i.e., scaffolds, devices), to encapsulate and protect biologically active products (i.e., cells, chemicals, and proteins), and to allow easy modification of chemical and physicochemical properties [5–10]. Following the implantation of the biomaterial, the healing process involves the interaction of many contributing factors. A variety of forces act upon the implanted biomaterial that can be generated either by the biomaterial itself or forces applied externally, ultimately signaling the cells to perceive and behave through the intracellular mechanosensitive machineries (Fig. 6.1) [11]. Potential challenges faced during the healing process are often attributed to the host response, despite technical advancements that aim at recovering the functionality of damaged organs and tissues. Thus, over the past few decades, numerous studies have contributed to discovering molecules and elucidating mechanisms in the intracellular mechano-transduction process [12–14]. Furthermore, it is evident that controlling these cell-biomaterial interactions seems to be crucial for successful therapeutic implementations [12].

The FBR (foreign body reaction) describes the host's inflammatory response to an exogenous material [15]. However, these implanted biomaterials have plagued the

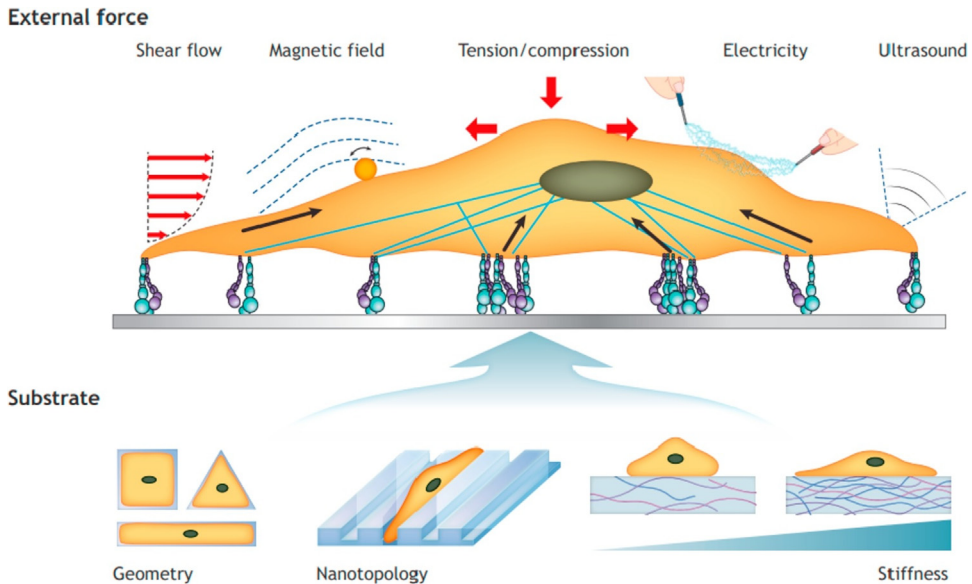


FIGURE 6.1 Different sources of physical cues triggering cellular mechano-responses: physical cues either forced externally (e.g., fluid shear force, mechanical tension/compression, magnetic vibration, electricity, ultrasound) or generated by biomaterials (e.g., geometry, nanotopography, stiffness) trigger cells to perceive environments and signal through intracellular mechanosensitive machineries that centered toward a nucleus. Source: Copyright Lee J-H, Kim D-H, Lee H-H, Kim H-W. Role of nuclear mechanosensitivity in determining cellular responses to forces and biomaterials. *Biomaterials* 2019;197:60–71.

clinical success, owing to the induction of adverse immune reactions resulting in excessive inflammation, impairment of healing, fibrotic encapsulation, tissue destruction, or even isolation and rejection of medical devices. The aforementioned scenario greatly mandates a deeper understanding of the material/biological environment interplay, in order to develop strategies and solutions to overcome the shortcomings which remain an existing challenge in the biomedical field. Hence, in the recent past, a critical factor under consideration is to choose materials that consolidate better with the tissues, without eliciting an FBR. Perhaps, biomaterial science has exerted ample efforts to understand the different cellular and molecular events characterizing biomaterial-immune system interactions. Researchers are now keen on biomaterials/designs that provide active cues to elicit a set of predefined cellular responses. Such responses may be improved cellular adhesion, migration and orientation, control of differentiation, or directing repair/regeneration.

This chapter provides an understanding on the current state of knowledge on the widespread mechanisms of FBR against biomaterials and also highlights on how the surface topography and physicochemical features of biomaterial surface can affect the quality and quantity of the reaction. Finally, it also outlines the recent researches that impart an insight into modification of specific features of different biomaterials that can control the inflammatory-immune response to implanted biomaterials and to promote tissue regeneration.

6.2 Host response—biomaterial interplay

Following implantation, biomaterial success and tissue regeneration are predominantly determined by the cellular and molecular responses of the human immune system, occurring at the interface between the foreign material and the host inflammation [16]. Primarily, the innate immune system is responsible for eliciting an immediate nonspecific inflammatory response on recognition of the biomaterial. The cells performing this function are the polymorphonuclear cells; mononuclear phagocyte cells namely the DCs (dendritic cells), monocytes and macrophages; and lymphocytes comprising of natural killer cells, gamma delta T cells, and innate lymphoid cell (ILC). On the other hand, the adaptive immune system exhibits a highly specific antigen response and thereby establishes a long-term memory. The performers of the adaptive response are the B and T lymphocytes. Fig. 6.2 highlights the key cells and proteins that orchestrate these responses that occur in a synchronized and tightly controlled way. Apparently, the biomaterial presence and its mechanics trigger a biological reaction with the development of an appropriate immune response that requires a well-orchestrated and controlled crosstalk between the aforementioned immune systems, by means of soluble factors and cellular subsets. Besides, the degradation products are released by them, such as tissue-engineered scaffolds, orthopedic implants, and biomedical devices; the resulting surface changes of the degrading biomaterials can also influence the immune system [17].

Many complex molecular and cellular players are involved in eliciting the FBR. The entire mechanism can be broadly classified into five sequential phases namely blood-biomaterial interaction, acute inflammation, chronic inflammation, foreign body giant cell (FBGC) formation, and encapsulation [18]. Unfortunately, the functionality and clinical success of the implanted biomaterial seems to be debilitated by the occurrence of this acute sterile inflammatory reaction, which obstructs tissue vascularization and remodeling with

Biomaterial implantation	
Immediate innate immune response: 0–4 h	• Blood protein precipitation (provisional matrix) • Coagulation, platelets • Complement • DAMPs, PAMPs
Induced innate immune response: 4 h to 4 days	• Tissue macrophages, PMNs • Monocytes, DCs, mast cells • ROS, IL-1β, TNF-α, IFN-γ, IL-12, CXCL8, IL-16, IL-8, MCP-1
Adaptive immune response: 4 days until resolution or healing	• B and T lymphocytes • Antibodies • TNF-α, IFN-γ, IL-2 • IL-4, IL-10, IL-13, TGF-β

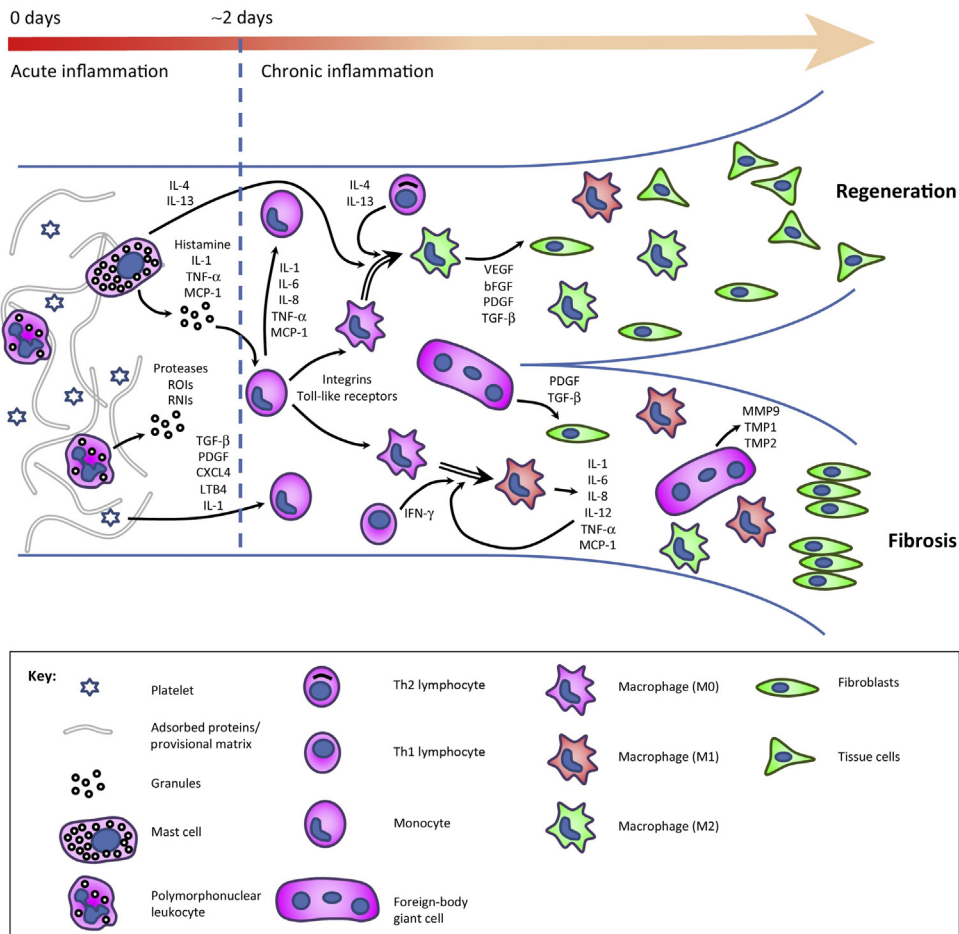
FIGURE 6.2 Overview of the cells and proteins involved in mediating effective immune responses. Source: Copyright Vishwakarma A, Bhise NS, Evangelista MB, Rouwkema J, Dokmeci MR, Ghaemmaghami AM, et al. *Engineering immunomodulatory biomaterials to tune the inflammatory response*. *Trends Biotechnol* 2016;34(6):470–82.

a fibrotic encapsulation that prevents further interplay between the biomaterial and the host tissue. However, FBR is the only feasible outcome of biomaterial implantation and extensive researches have elucidated that the modulation of this response can significantly contribute to implantation success [1]. Fig. 6.3 provides an illustration of the multiple phases of immune responses involving the key players and events, that can either result in fibrous tissue repair and encapsulation or seldom observed complete regeneration.

6.2.1 Phase I

Within a few seconds from implantation, blood from the damaged vessels surrounds, and begins interacting with the biomaterial. Over the next few minutes, the biomaterial surface displays active and spontaneous protein adsorption, involving host plasma components such as proteins (albumin, fibrinogen, fibronectin, vitronectin, and gammaglobulins), lipids, sugars, and ions [6,19]. Researchers describe this premature phase of protein adsorption by Vroman effect [20], which is most common on hydrophilic surfaces that contain loosely-bound proteins [21]. According to this theory, proteins with higher mobility like albumin are adsorbed foremost and spontaneously followed by less motile proteins with higher affinity for specific surfaces like high molecular weight kininogen (HMWK), fibrinogen, fibronectin, and vitronectin [6,22,23]. This sparse provisional (protein) matrix is formed by proteins that have high affinity to biomaterial surfaces [18] and is measured approximately 2–5 nm [24]. During the following hours/days, the process of recruitment and adhesion of tissue-derived, inflammatory, vascular, and stromal cells takes place. Researchers have shown that the various characteristics of the biomaterial surface (such as energy, chemistry, topography, and roughness) significantly influence the type, amount, composition, and conformation changes of the adsorbed molecules [6]. Thus, these characteristics serve as potential determinants of the tissue reaction to the implanted biomaterial [6,25].

Besides proteins, lipids, and sugars, the blood exudate also contains platelets and other components of the coagulation cascade, and the resulting clot formation defines the provisional matrix around the biomaterial [23,26]. The provisional matrix can release diverse amounts of chemoattractants, cytokines, and growth factors, thereby influencing the FBR by regulating the attraction and activity of macrophages and other immune cells. Anderson et al., hence, described the provisional matrix as “a naturally derived, biodegradable sustained release system in which bioactive agents are released to control subsequent phases of wound healing” [17]. The concerned cells begin communicating with the provisional matrix instead of the biomaterial surface, and the constitution of the provisional matrix is considered to be of utmost relevance for all the cascading episodes of the FBR [27]. The provisional matrix subsequently leads to further platelet activation and aggregation [28,29] and also stimulates the fibrinogen cleavage into fibrin under the influence of thrombin [30]. According to a few hypotheses aiming to substantiate the conversion of fibrinogen to fibrin, the biomaterial surface-promoted autocatalytic activation of FXII on negatively charged anionic surface initiates the intrinsic coagulation system [23,31,32]. Nevertheless, current concepts propose that the FXII activation is facilitated by a composite protein adsorption competition effect in the fluid phase on the biomaterial



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FIGURE 6.3 Overview of the immune response to a biomaterial : implantation of a biomaterial, and the accompanied damaging of blood vessels, triggers an acute inflammatory response. This phase starts with the absorption of proteins to the surface and the formation of a provisional matrix. The matrix contains a high amount of platelets that plug the injured blood vessels, but also serve as a reservoir for growth factors and cytokines. This environment results in the recruitment of polymorpho- nuclear leukocytes and mast cells, which characterize the acute inflammation phase. A range of growth factors are subsequently secreted by the platelets and through degranulation of the neutrophils and mast cells, which are key players to recruit macrophages to the site of implantation. The following stage is the chronic inflammatory phase defined by the presence of mononuclear blood monocytes that can differentiate to become macrophages upon activation. During the initial phase of chronic inflammation, there is an increase in specific proinflammatory (M1) macrophages in the injured site. Based on, among others, stimulation by Th lymphocytes, the polarization of the macrophage population can change to a pro-healing phenotype (M2). When this switch is made, tissue cells are stimulated toward regeneration of the implant site. However, if the environment fails to induce the switch to an abundance of M2 macrophages, the chronic inflammation phase will lead to fibrosis. *Th*, T helper. Source: Copyright Vishwakarma A, Bhise NS, Evangelista MB, Rouwkema J, Dokmeci MR, Ghaemmaghami AM, et al. *Engineering immunomodulatory biomaterials to tune the inflammatory response*. Trends Biotechnol 2016;34(6):470–82.

surface [33]. When thrombin splits fibrinogen to fibrin, to form a classic mesh-like fibrillary coat on the surface of the transplanted biomaterial, not all of the fibrinogen is transferred to fibrin. The non-transformed fibrinogen undergoes an adhesion-mediated conformational change [34] that renders it capable of interacting with α -integrins on platelet membranes, thereby resulting in platelet adhesion, activation, and ensuing aggregation of further platelets [17,35,36]. It should be noted that the adherent fibrinogen and von Willebrand factor (vWF) also serve as potential adhesion matrix for macrophages [37]. Furthermore, the complement proteins that are activated upon contact with the biomaterial, synergistically reinforce a chain of events such as platelet adhesion and activation [38–40], recruitment, and adhesion of additional immune cells [41,42]. These immune cells are eventually attracted by the local bulk of proinflammatory cytokines, chemokines, and growth factors [17,43].

Following the establishment of the provisional matrix, the acute and chronic inflammatory responses follow each other. The implanted biomaterial and the amount of the surgical trauma caused often determine the intensity and duration of the acute and chronic inflammatory responses [17]. Findings have reported that the extracellular matrix (ECM) adhesion proteins, namely fibronectin, and vitronectin also adhere to biomaterial surfaces [44] and essentially modulate the inflammatory reaction to biomaterials. On the contrary, fibrinogen and complement system are predominantly involved in activating the cellular component of inflammation. Additionally, fibronectin and vitronectin respectively enhance cell adhesion [45–47], promote macrophage fusion, and participate in the FBR chronic phase [48–50].

6.2.2 Phase II

The infiltration of polymorphonuclear leukocytes (PMNs) and mast cells induces the following acute inflammation phase of FBR against biomaterials [17]. The majority of the biomaterials such as hydrogels and polymers are reported to be inert and no-toxic to this short-lived phase (hours to a few days long), despite the findings that reveal continuous chronic infiltration of macrophages, which mandates further research [51]. According to the literature, the acute inflammatory phase comprises of three key mechanisms that are held responsible for leukocyte migration, adhesion, and activation: (1) tissue damage during implantation, (2) recognition and interaction with the provisional matrix, and (3) direct recognition of the biomaterial.

The destruction/degradation of the biomaterial is often attempted by the activated neutrophils which are recruited from the peripheral blood by chemoattractant factors (released from host activated platelets, endothelial cells, and injured tissue cells), and primarily get adhered to the implantation site (by means of β 2-integrins). The integrin signals mediate cell adhesion to protein-coated biomaterials and their subsequent activation [7,52,53]. The biomaterial may undergo degradation either through phagocytosis, or by proteolytic enzymes, or by reactive oxygen species (ROS) released by cytoplasmic granules [54–57]. Concurrently, the leukocytes undergo activation by means of some pattern recognition receptors (PRRs) [usually interacting with pathogen-associated molecular patterns (PAMPs), found on microorganisms and primarily expressed on

macrophages and DCs] [58,59]. They induce immune responses driven by molecules within the family of damage-associated molecular patterns. Besides, certain chemoattractants like coagulation factor VII, XI, platelet factor IV (PF4), vWF, or P-selectin are also released by the endothelial cells, activated platelets, and the complement system. This potentiates the further attraction of PMN and succeeding macrophages to the site of implantation [38,60]. Furthermore, few endogenous equivalents of PAMPs namely the alarmins, which are recognized by macrophages and DCs, through PRRs (here acting as scavenger receptors), toll-like receptors (TLR), and C-type lectin, also participate in the leukocyte activation. These Alarmins basically comprise of heat shock proteins, high mobility group box 1, adenosine triphosphate, and uric acid [61–63]. Current studies have shown that the interaction of endothelial cells with leukocytes and the mechanisms of vascular permeability may also have an effect on the progress of the FBR [64–66].

In addition to the aforementioned mechanisms, neutrophils release neutrophil extracellular traps (NETs) [67], a “sticky network” of granular proteins, neutrophil elastase, chromatin DNA, and histones [68], usually involved in trapping pathogens and preventing infection spread [67]. The progressive release of these extracellular traps is capable of prolonging the fibrotic tissue response, thereby leading to the excessive production of a dense fibrotic matrix [69]. The undue production of NETs prevents integration between the tissue and the biomaterial and degrades neutrophil-produced cytokines and chemokines that regulate the healing process [69,70]. This impairs the healing response and the potential for tissue regeneration, promoting fibrotic encapsulation [71]. Furthermore, NETs release from neutrophils, unable to phagocytose a harmful stimulus [72], may be considered similar to the formation of FBGCs by the fusion of frustrated macrophages [73]. Besides neutrophils, mast cells also play a key role in acute inflammation. Subsequent attraction of more leukocytes and macrophages are effected by the mast cell-derived histamine, interleukin (IL)-4, IL-13, and PMN-derived IL-8, macrophage chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)1 β , eventually progressing to the chronic inflammation phase [74–76]. Moreover, frustrated phagocytosis of the neutrophils and oxygenic burst initiates a predominant proinflammatory milieu that accelerates tissue degeneration, consequently attracting more PMN, and thereby initiates chronic inflammation [54].

Current knowledge states that the proteins present inside and on the surface of the provisional matrix are recognized as a danger signal. Several α -integrins and TLR on leukocytes recognize fibronectin and vitronectin which are adsorbed to the surface [77]. Furthermore, the conformational changes of biomaterial-adsorbed fibrinogen and HMWK sets free the α M β 2-integrin binding sites for PMN and macrophages [78,79]. Perhaps, it is understood that the interaction of PMN and macrophages with the provisional matrix constitutes the major factor during inflammation. Additionally, it should be noted that the shape of the implant has an influence on the inflammatory response and capsule formation. In the case of scaffolds, the capsule thickness and inflammatory infiltration cells are found to be significantly decreased on days 7–28, whereas for films constructed using the same polymer, it remains unchanged [80]. Some biomaterials are reported to be less inert and nonimmunogenic, and thereby are directly recognized by TLR or via identification of hydrophobic portions of biomolecules [81].

When activated, the neutrophils synthesize a remarkable amount of immune-regulatory signals [74]: CXCL (CX chemokine ligand)8 (the most prominent chemokine),

CCL (C chemokine ligand)2 and CCL4, both potent chemoattractant and activation factors for monocytes, macrophages, immature DCs, and lymphocytes [82]. The primary targets of these chemokines are neutrophils themselves and their progressive increase fosters monocyte infiltration and suppresses neutrophils recruitment. Thus, lack of subsequent activating signals persuades the neutrophils to undergo apoptosis, thereby disappearing from the implantation site [17].

6.2.3 Phase III

Biomaterial research describes chronic inflammation as a phase distinguished by the infiltration of monocytes and lymphocytes, lasting for a brief period of 2–3 weeks (weeks 2–5 after implantation) [83]. During this stage, but also earlier and in parallel to neutrophil recruitment, the circulating monocytes respond to PMN-, platelet-, and mast cell-derived chemoattractant (such as CCL2, CCL3, and CCL4) [17] and bind fibrinogen in the biomaterial provisional matrix, thereby undergoing activation [50,84,85] (43,64,65 of 10) and differentiation into the classically activated or “M1” macrophages [15,49,86,87]. These cells have been classified according to their ability to secrete proinflammatory cytokines [such as IL-1 β , IL-6, tumor necrosis factor (TNF)- α], chemokines [86,88], and enzymes.

The complete process (initiation, duration, and outcome) of the host response against the biomaterials is majorly governed by the monocytes/macrophages [81]. Factors like complement factors, transforming growth factor (TGF)- β , platelet-derived growth factor (PDGF), PF4, MCP-1,2,3,4, RANTES, MIP-1 α , and MIP-1 β are responsible for the attraction of these cells to the implantation site [89,90]. Additionally, they also directly recognize biomaterials or biomaterial-associated proteins by TLR and scavenger receptors [29].

Following entry via the β 1-, β 2-, and β 3-integrin receptors the macrophages gain adhesion to fibrinogen, complement fragments, fibronectin, and vitronectin of the provisional matrix [91,92]. The circulating monocytes respond to chemoattractant (such as CCL2, CCL3, and CCL4) and the consequent binding at the site of injury further triggers the activation of these monocytes, thereby undergoing further differentiation into the classically activated or “M1” macrophages [15,49,86,87]. The characteristic feature of M1 macrophages is their ability to secrete proinflammatory cytokines, (such as IL-1 β , IL-6, TNF- α), chemokines [86,88], and enzymes. Furthermore, the adherent macrophages secrete chemokines (CCL2, CCL4, CXCL8) [88] that potentiate the invasion of additional inflammatory cells and also attempt to degrade the biomaterial by releasing ROS and degrading enzymes. As a result of the biomaterial being too large to be internalized, they undergo “frustrated” phagocytosis causing a surge in the release of cytokines from mast cells or T helper (Th)2 lymphocytes, namely the IL-4 and IL-13. These macrophages eventually shift to the antiinflammatory, “M2” phenotype [93]. This shift triggers the secretion of antiinflammatory cytokines (such as IL-10 and TNF- β) that can reduce the degradative capacity and enhance the tissue remodeling activity via matrix metalloproteinases; [94,95] and potentiate the induction of migration and proliferation of fibroblasts toward effective tissue regeneration. Results of some studies emphasized that the macrophages in the FBR may display varying phenotypes with features of both M1 and M2 polarization [60]. However, a few studies were able to report significant associative findings between higher

percentage of M2 at the implantation site and tissue integration, diminished scar tissue formation, and increased neovascularization [54,74,75].

During the chronic inflammatory phase, cytokines are mainly produced by directly or indirectly activated T lymphocytes, mainly CD (cluster of differentiation)4 helper T cells and their Th1 and Th2 subsets. Extensive production of these cytokines results in modulation of the pro/antiinflammatory responses [96]. The interrelationship between the M1/M2 macrophage phenotype and the altered cytokine expression profile from Th1 to Th2 lymphocytes emphasize on the crucial role played by T lymphocytes in promoting resolution of inflammation and regeneration. Studies have elucidated that the T cells attach to the biomaterial [97], become activated through noncanonical pathways and enhance further macrophage adhesion and fusion into FBGCs through paracrine actions of secreted cytokines.

6.2.4 Phase IV

The overlapping events of the phenotypic M1 to M2 switch as well as the mechanisms of frustrated phagocytosis, result in macrophage membrane fusion to form a FBGC on the biomaterial surface, a hallmark of chronic inflammation. The FBGC which are several hundred nm large with several dozens of nuclei remains resolute as long as the biomaterial is identified in the subcutaneous tissue [98]. Perhaps, the formation of FBGC is a consecutive attempt to enhance their phagocytic functionality and ignore anoikis and apoptosis [99,100]. Owing to the long-term adherence of the FBGC to the surface of the biomaterial, it forms a barrier between the tissue and device, subsequently resulting in implant deterioration and/or loss. Therefore, the macrophage plasticity renders them adaptable to immune-regulatory, host defense, tissue repair roles in response to the implant properties.

The formation of FBGCs is often a signature component of biomaterial-induced FBR and is encouraged through the activation of basophils, mast cells (release histamine), and Th cells that secrete IL-4 and IL-13, thereby enhancing the macrophage fusion on biomaterials. Studies have identified IL-4 and IL-13 as the most potent ambient signals regulating the fusion of “frustrated” macrophages [78,101–103]. Besides, the resultant FBGC is further characterized by the expression of CD11, CD45, and CD31 membrane proteins and receptors for IL-1, IL-2, IL-4, and IL-8 [117]. Nevertheless, it is understood that the FBGC formation at the biomaterial surface is undesirable. The reason being that, over a longer period of time, the FBGC favors many bioreactive agents like ROS, degradative enzymes and acids, eventually resulting in biodegradation of the implanted material, and device failure [27,91]. As a measure to prevent this adverse effect against the implanted medical devices, in a recent study antioxidants were either incorporated within or on the surface of the material, to moderate oxidation [104].

6.2.5 Phase V

Recruitment of fibroblasts are initiated by the profibrogenic factors such as PDGF [105], vascular endothelial growth factor (VEGF), and TGF- β , that are released by the coordinated action of the immune cells. The activated fibroblasts deposit collagen (type I and III)

in an attempt to repair the damaged tissue. However, the uncontrolled collagen secretion leads to an undesirable fibrotic deposition of ECM, which encapsulates the biomaterial and further compromises the implant function. Thus, institution of chronic inflammation and FBGC formation ultimately results in the formation of a fibrotic, collagenous capsule around the biomaterial. Besides fibroblasts and immune cells, the keratinocytes, endothelial cells, thrombocytes, and adipocytes also have a role in capsule formation [99,106,107]. In addition, the ECM remodeling around the implanted biomaterials is also affected by the proteolytic enzymes such as matrix metalloproteinases (MMP) secreted by macrophages and/or endothelial cells. In a study by Binnebosel et al., inhibition of MMP-2 has been shown to attenuate FBR against polyvinylidene fluoride meshes in mice [108].

During the proregenerative phase, the M2 macrophages which possess antiinflammatory/antifibrotic phenotype, conduct a crosstalk with a subpopulation of T cells defined as regulatory (Tregs), which play a pivotal role in tissue immune homeostasis. These cells can skew the local immune response from inflammation to a proregenerative tissue repair cascade, sustaining the antiinflammatory/antifibrotic phenotype by the secretion of antiinflammatory cytokines, such as IL-10. Furthermore, Tregs are able to improve healing quality by inducing a type II response, including antiinflammatory macrophages. Following a T cells decrease, resident Treg levels remain elevated, probably because they display an epithelial growth factor receptor [109,110] whose expression allowed the growth factor amphiregulin secreted by mast cells to maintain Tregs at the damaged site [109]. Once present, Tregs proliferate and upregulate the secretion of amphiregulin, which may induce either cell proliferation [111] or differentiation and is necessary for regeneration. Tregs may also enhance the regenerative capacity of endogenous stem/progenitor cells through the secretion of growth factors.

In addition to the aforementioned effects, the fibroblasts and endothelial cells at the biomaterial surface result in granulation tissue which is formed by the deposition of collagen and other ECM proteins. The granulation tissue basically consists of a loose network of collagen fibers, proliferating capillary sprouts, fibroblasts that secrete collagen, and phagocytosing macrophages [112]. Subsequent maturation of the granulation tissue renders it less cellular and more collagenous (eventual replacement of type III by type I collagen), transforming it into a peripheral fibrous capsule that partially/completely prevents the biomaterial from interacting with the surrounding tissue [51,60,113]. During this stage, some fibroblasts are reported to undergo differentiation into myofibroblasts by TGF- β , which further contracts the capsule, entailing deformation, mechanical stress, and aesthetic problems [51,74]. Thus, fibroblasts and myofibroblasts being the key players in fibrotic reaction execute the deposition of new matrix. It is further believed that the contraction of the myofibroblasts is responsible for the fibrotic scar formation. In terms of normal wound healing, this process ends with the resolution phase which is distinguished by apoptosis and senescence of myofibroblasts and fibroblasts, regression of the neovasculature, and decline in collagen caused by a certain group of fibrinolytic macrophages. Whereas during FBR, this resolution phase is found to be missing, presumably because of the persisting initiating agent and biomaterial, with ongoing proinflammatory or profibrotic stimulation of cells near the biomaterial.

6.3 Other significant players of the foreign body reaction

During the interaction between the biomaterial and host response, it has been reported that a few other cells and mechanisms also play an important role in resolution of inflammation and successful regeneration process.

1. *Complement system*: Besides the coagulation system, the complement system also significantly determines the fate of the implanted biomaterial. Failure of the implanted biomaterial can also be attributed to complement activation that subsequently boosts the immune reaction and plasmatic coagulation [114]. It is believed that the nonspecifically bound immunoglobulins present in the provisional matrix binds the C1 subunits and activates the C3 convertase, leading to the activation of the classical complement system pathway [23,115]. However, C1q can also directly bind to the biomaterial surface. Presence of carboxyl, sulfate, hydroxyl, and amino groups on the biomaterial surface can bind C3b, activating the alternative pathway. Further, the bound C3b activates the C3 convertase, which promotes a positive amplification loop and finally activates the downstream C5 convertase [23,77,114,116]. Nevertheless, the induction of the complement system in cases where the biomaterials did not possess the mentioned surface characteristics needs further understanding [23]. During this activation process, excessive amounts of C3a and C5a generated serve as powerful chemoattractants for phagocytes and prompts the degranulation of mast cells and neutrophils. On the other hand, C3b present on the provisional matrix acts as a ligand for leukocyte adhesion via integrin receptors [77]. Overall, the activation of the complement system acts as a driving force during events such as acute or chronic inflammation, thrombotic complications, as well as encapsulation with a feasible loss of function of a device [17,23].
2. *DCs*: These cells are seen to phagocytize particles and process danger signals at the injury site, similar to macrophages. Even though their specific role during tissue repair and regeneration is not well-determined [117], studies have shown that DCs play a significant role in the tissue healing process. These cells possess immune-regulatory actions and they are seen to interact with T cells and B cells to initiate and shape the adaptive immune response, and also induce the activation and growth of regulatory T cells (Treg). Additionally, they also influence the development of tolerogenic or anergic T cells, depending on their maturation stage, location, and cytokine environment [118]. Current studies have elucidated the novel “adjuvant” role of biomaterial under an immunogenic stimulus, which was triggered during the interaction between DCs and biomaterials. It was studied that in the presence of an immunogenic stimulus, there was an interaction between DCs and biomaterials and the increased immune responses to co-delivered antigens [119]. However, it remains an assumption that only the direct contact between the cells and material can activate the DCs [120]. It is indeed interesting to note that the biomaterials prime DCs through PRR signaling pathways [61], and the type of the PRR triggered determines the subsequent mechanism. For instance, maturation of the DCs result in lengthening of the immune response to biomaterials, whereas inhibition contributes to delayed wound healing or down-regulation of the inflammatory response [121].

3. *Th17 cells*: Besides the Th1 and Th2 cell paradigm, a distinct T cell effector subset namely the Th17 was identified, mainly responsible for the production of IL-17 and IL-22. Th17 cells along with its cytokines are the basic components of the mucosal immune system and any alteration is closely linked to autoimmune and inflammatory diseases [122]. A recent report revealed that the differentiation of monocyte/macrophage populations to a profibrotic phenotype was effected by IL-17 mediated signaling. However, IL-17 is redundantly produced by other immune cell populations, including $\gamma\delta$ T cells (122 of 10) and ILCs type III (ILC3) [123].
4. *$\gamma\delta$ T cell subset*: Displaying a prevalent surveillance role in native tissue [124], unlike the $\alpha\beta$ T cell subset that possesses both pro and antiregenerative characteristics, the $\gamma\delta$ T cell subset is reported to exhibit proregenerative properties. Although studies in mice and humans have demonstrated their beneficial role in healing of skin wounds, their potential proregenerative role in the field of biomaterials is yet to be elucidated.
5. *CD8 cytotoxic T lymphocyte (CTL) cells*: Studies have reported that CTL cell subset has the ability to influencing wound healing, as demonstrated by the improvement of its outcome following CD8 T cell depletion in rats [125] and by the negative impact on bone fractures, following a CD8 T cells increase in humans [126].
6. *B cells*: Likewise T cell depletion, scientific data reveals the role of B cells in tissue healing. One study pointed out that adaptive immune system deficient mice exhibit faster bone healing [127], thereby showing that the depletion of B cells served as a promising strategy to augment bone regeneration. Its role in the repair and regeneration of other tissues is still under study.
7. *ILCs*: Recent data reveals that a lack of expression of T or B cell receptors defines the production of the (ILCs). They can further be subdivided into three classes (ILC1, 2, 3), characterized by their canonical transcription factors and cytokine expression [128]. These subsets somewhat mirror the expression of Th1, Th2, and Th17, respectively. Similar to Th2 cells and M2 macrophages, ILC2 is antiinflammatory in nature producing IL-4, IL-5, and IL-13. Besides providing a set of cell signaling mediators and metabolites which are associated with wound healing, ILC2 also promotes CD4 T cell polarization toward Th2 cells via positive inhibition of Th1 [129–131]. Considering that the development of a proregenerative response to biomaterials requires type II cell populations [132] and that the crosstalk Th2/ILC2 is central to tissue proregenerative responses, ILC2 activity may be relevant with biomaterials.

The activation and varied actions of the aforementioned immune cells and the mutual crosstalk between the different innate and adaptive cellular components provide a deeper insight into the significance of the design of biomaterials, which can possibly tune the immune response to their benefit [27].

6.4 Impact of biomaterial surface characteristics on the sequential phases of host response

6.4.1 On protein adsorption

This is an intricate process that relies on varied properties of the biomaterial surface like wettability, topography, elasticity, chemical composition, and charge. Table 6.2 summarizes

some of the main chemical characteristics and their involvement in the immunological response. Nevertheless, the protein characteristics such as structure, isoelectric point, and relative concentration in the plasma and protein- surface affinity also have an influence (Table 6.1). Parameters like dehydration of the protein and the surface, redistribution of charged groups in the interface and conformational changes in the protein molecule are often affected by the surface characteristics [6]. Researchers focused hard to understand the underlying mechanisms, which can allow them to influence the composition of the provisional matrix to conquer clinical success [6,35,46,77]. According to significant studies in the literature, several materials were reported to express reduced protein adsorption, due to their inherent physicochemical properties like wettability, roughness/topography, elasticity, and surface charge.

Surface wettability (i.e., hydrophobicity or hydrophilicity) is a key requisite for protein adsorption on biomaterial surfaces [6]. Basically, hydrophilic surfaces contain tightly bound water molecules, thereby establishing a steady water layer on a surface that can serve as a barrier for surface-protein as well as cellular interactions [133,134]. On the other hand, owing to easy replacement of water molecules from the surface, hydrophobic

TABLE 6.1 Surface chemistry: commonly explored chemical moieties.

Groups	–NH ₂ (amino)	–OH (hydroxyl)	–COOH (carboxyl)	–CH ₃ (methyl)
Surfaces	Hydrophilic	Hydrophilic	Hydrophilic	Hydrophobic
Charges	Positive	Neutral	Negative	Neutral
Focal adhesions	Medium	High	Medium	Low
Ability to access fibronectin domains, integrin binding, cell adhesion	Medium	High	Medium	Low
Inflammatory cell infiltration	High (in vivo)	High (in vivo)	Low	High (in vitro)
Macrophage response	Antiinflammatory	Low inflammatory	Inflammatory	
			Low inflammatory	
			Low inflammatory promoting regulatory T cell phenotypes (mouse model)	
Thickness of fibrotic capsules around the implant	High (in vivo)	High (in vivo)	Low	High (in vitro)
Cell differentiation pathways	Medium (osteoblasts)	High (osteoblasts)	Medium (osteoblasts)	Low (osteoblasts and myoblasts)

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surfaces normally adsorb more proteins than hydrophilic surfaces [103]. However, some studies have reported similarity in the adsorption capacities of the hydrophilic and hydrophobic surfaces [74,78,91,102,104,135]. Surface charge and protein conformation are factors that are found to have better relevance than surface wettability for some proteins on certain surfaces [101]. Both fibrinogen and vitronectin have a better affinity for positively or negatively charged hydrophobic surfaces as compared to uncharged hydrophobic surfaces. Additionally, recent findings have shown that pH and small ions in the aqueous solution can also influence the surface charge [135].

Surface topography (general roughness), is another feature that often is associated with an increased protein adsorption, cell adhesion, and differentiation. A study by Besenbacher et al. revealed that nanorough surfaces (surface features smaller than 100 nm) have the ability to modify protein conformations [136]. Proteins with dimensions of the same order as the surface were not conformationally altered, while proteins with dimensions much smaller or larger than the surface roughness were seen to be altered upon adsorption. It is hypothesized that these changes are a result of roughness-mediated confined spaces that restrain with the wettability of the surface or may intensify the surface energy to adsorb proteins [94,137]. A study by Mooney et al. showed that placing parallel grooves on silicone substrates caused an even orientation of fibronectin, vitronectin, and dermal fibroblasts [138]. Furthermore, the rough and hydrophobic surfaces promote the formation of stable gas nucleoli, even in the smallest micropores. When these entrapped air nuclei are not thoroughly removed from the surface, it may lead to thrombogenicity.

Other significant factors that are elicited by body movements at the implant interface are shear stress and strain. Mechanical stress is a well-recognized stimulator of different cells. Griendling and FitzGerald reported that all cell types tested to date responded to shear stress. Whenever there are increased strain levels at the interface, it triggers the production of proinflammatory signals that recruit immune cells, eventually leading to FBR [139,140]. Even a higher elastic modulus can create a stress at the interface, eventually activating a proinflammatory signal that leads to the formation of a fibrotic capsule, or what is referred to as “poor biocompatibility” of the material [141]. Hilborn and Bjursten, thereby emphasized the importance of decreased unfavorable mechanical stresses around the implant, which can be done by designing the biomaterial to have elastic modulus that can adapt to the elasticity of the tissues [141].

6.4.2 On acute inflammation

Literature presents limited data on the impact of biomaterial properties on the acute phase of inflammation. Some essential predisposing factors for acute inflammatory phase includes the amount of tissue damage during implantation, the quality and quantity of protein adsorption, and contamination of the implant. It is often assumed that the provisional matrix serves as a barrier preventing the direct contact between infiltrating early leukocytes and the biomaterial. However, studies have found that biomaterials in a protein-free setting also induce similar inflammation as protein-covered biofilms [77,81,142]. Thus it is understood that PMN and macrophages may directly recognize the biomaterial surfaces during

the early phase of inflammation. Bryers et al. showed that the hydrophobic micro or nano areas of the biomaterial were recognized by TLR or activation of scavenger receptors [81].

Perhaps, most of the molecules in the provisional matrix are recognized by TLRs. However, it was found that TLR4 can be stimulated directly by some cationic polymers like polylysine, polyethyleneimine, cationic dextran, and cationic gelatin [143]. Thus, identification of the hydrophobic parts of the biomaterial that can initiate the activation of TLR seems to be another mechanism of biomaterial recognition [144]. It was found that the unspecific bonds which bind substances like alarmin attached to the biomaterial, also promote binding and activation of TLR2 and TLR4. Nevertheless, a few interesting researches reported that biomaterial structures such as hydrophobic polypropylene oxide, hydrophilic polyethylene oxide regions, oxidized alkane polymers, and polystyrene can directly activate TLR1,2,4,6 in a protein-free setting [104,137,142,145,146]. Nevertheless, data on the association between surface topography (like roughness) and acute inflammation is limited.

6.4.3 On chronic inflammation

Numerous studies have studied the influence of biomaterial surface chemistry and topography on inflammation, macrophage infiltration, adhesion, and activation. A recent finding revealed that unlike the hydrophilic, anionic surfaces, the hydrophobic, cationic surfaces promote macrophage adhesion [83]. Additionally, it was also reported that nickel, magnesium, corroding metals in general as well as hydroxyl and amino groups may stimulate a more intense inflammation, with increasing numbers of infiltrating macrophages and lymphocytes.

The three-dimensional surface topography also has a significant effect on chronic inflammation. Table 6.2 demonstrates some of the effects induced on cells by different particle sizes. Besides size, the shape of the biomaterial is also a significant parameter that can have an effect on its interaction with immune cells (Table 6.3). Studies showed that the biomaterials with pore sizes around 30–40 μm were seen to be associated with the highest number of infiltrating macrophages, a higher portion of M2 macrophages, and the highest vascularization and best healing success [81,147]. Mohiuddin et al. showed that 50 nm nanodots on an aluminum oxide-based array increased IL-6 secretion, adhesion, density, and the spread of macrophages when compared to flat surfaces or very large dots. However, their influence on the final outcome of the reaction which is the FBGC and capsule formation remains unknown [148]. Hilborn and Bjursten showed that increased amount of inflammation was reported whenever there was a mismatch of a stiff material, low tissue stiffness, and sharper edges on triangular-shaped biomaterials, which can be attributed to higher mechanical irritation [141].

6.4.4 On foreign body giant cell formation

Besides environmental signals and the presence of fusogenic molecules on the biomaterial surface, the quantity and quality of the adsorbed proteins in the provisional matrix,

TABLE 6.2 Biomaterial topography: size.

Size	Cell types	Findings
Nano scale	Platelets	36 nm particles: induced activation and cell flattening 56 nm particles: decreased platelet activation
	Macrophages	- Smooth surfaces 50–200 nm nanodots: increased IL-6 secretion 50 nm nanodots: induced maximum cell spreading, focal adhesion, cell density - Reduced migration and activation on nanostructured titanium - Inhibition of iNOS, NO, and proinflammatory cytokines - Decreased migration on surfaces
	Dendritic cells	3 nm: enhanced activation, increased IL-12 and IFN-γ production - Increased proinflammatory T cell activity in co-culture 12 nm: increased IL-4 secretion
Nano-submicronscale	Macrophages	- Skewed T cell immune responses toward wound healing - Reduced initial adhesion on titanium surface - Less differentiated morphology - Reduced adhesion and proinflammatory cytokine release
Micron scale	Macrophages	- Reduced adhesion and proinflammatory cytokine release - Reduced inflammatory cytokine secretion - Protected cells from M1-inducing stimuli LPS and IFN-γ
		2–40 μm particles: size-dependent production of IL-10 and TNF-α - Involved TLR2 stimulation - Largest particles: did not induce cytokines
Meso scale	Macrophages	0.5 mm diameter particles: intraperitoneal fibrotic growth (mouse) 1.5–2 mm diameter particles: reduced fibrotic tissue formation in mice and non-human primates - Medium particles biased responses toward M1 inflammatory phenotypes - Larger particles caused a shift toward M2 immune-regulatory and wound healing phenotypes

IFN- γ , Interferon gamma; IL, Interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; TLR, toll-like receptor; TNF- α , tumor necrosis factor- α .

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the surface itself, and topographic features also determine the extremity of the FBR and FBGC formation. Amongst the proteins present on the biomaterial surface, the adsorbed vitronectin, and fibronectin (to a lesser extent) are also responsible for the fusion of macrophages [17,149]. It was reported that compared to hydrophilic and hydrophobic cationic surfaces the hydrophilic anionic, and nonionic polyacrylamide/polyacrylic acid surfaces demonstrate diminished monocyte adhesion and FBGC formation [83].

When it comes to surface topography, it was understood that the smooth, flat surfaces induce considerably more FBGC formation than the rough surfaces [27]. Additionally, it was reported that the larger polylactic glycolic acid (PLGA) microspheres (30 μ m) induce more FBGC than smaller (6 μ m) microspheres [150]. In the case of materials like hydrogels, ceramics, metals, and plastics, sphere size larger than 1.5 μ m induced less fibrosis as compared to spheres of various smaller sizes [151].

TABLE 6.3 Biomaterial topography: shape.

Cell types	Findings
Macrophages	• Internalization of gold nanorods was stronger compared to that of nanospheres owing to preferential uptake of the former via micropinocytosis • The shape dependence of macrophage behavior was investigated by testing these cells with rods of varying lengths • Shorter rods were more rapidly internalized • Longer rods induced enhanced inflammatory mediators (IL-1α and TNF-α) since not readily phagocytosed • The different shaped cross sections of rods extruded from medical-grade materials affected the FBR extent: circular cross sections induced the least-extensive reaction compared to pentagonal and triangular ones • Smooth surfaces led to less acute reactions than sharp; corners, acute angle surfaces
Neutrophils	• The rough rather than smooth surface of polystyrene-polyethylene oxide particles boosted neutrophil recruitment and IL-1β production • Rough particles: preferentially taken up by macrophages, increased activation of inflammation
DCs	• Titanium dioxide shaped as particles (diameters of 7–10 or 15–20 nm), or as nanotubes (diameters of 10–15 nm and lengths of 70–150 nm): induced shape dependent cytokine secretion, reactive oxygen species production, DCs maturation • The shape dependence of DCs response was confirmed with antigen-coated gold spherical, rod-shaped or cubical nanostructures that elicited differential cytokine secretion and antibody production • Rod-shaped particles induced IL-1β, spherical and cubical ones induced TNF-α, leading to a less specific inflammatory response
T lymphocytes	• Collagen ECM scaffolds: critical role of Th2 cells in wound healing, induced a regenerative microenvironment • Supporting role of T CD8 and B cells

CD, Cluster of differentiation; DC, dendritic cell; ECM, extracellular matrix; FBR, foreign body reaction; IL, interleukin; Th, T helper; TNF- α , tumor necrosis factor- α .

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6.4.5 On capsule formation and fibrosis

According to current concepts, four prevailing approaches are observed to reduce capsule formation or fibrosis, respectively: (1) physical, chemical, and topographical biomaterial surface modification, (2) alteration of the systemic immune reaction, and (3) alteration of the local immune reaction. DiEgidio et al. concluded that to date no study confirmed the efficiency of a systemic treatment to eliminate fibrous capsule formation. However, a few studies found a mild decrease in capsule thickness, but it was not sufficient to compensate for the induced systemic side effects [113]. In contrast, local treatment like molecular coatings, incorporation of modifying agents in the biomaterial, and local application of drugs into the implantation site were found to be more efficient [152]. It was reported that locally released or administered steroids or TGF- β inhibitors have an initial impact on FBR and capsule formation. Nevertheless, in most cases the long-term kinetics have not been analyzed or a decline of the drug due to dilution and metabolism has been observed. As a consequence, impaired wound healing and proper integration of the device into the tissue occurred with these anti-inflammatory effects [113]. Recent research proposed therapeutic interventions to accelerate the portion of M2 remodeling subtype macrophages in

the lesion by targeting CCR1 and CCR2 on M1 inflammatory macrophages or the transfer of autologous M2 macrophages into the lesion [153,154].

A great many studies on the biocompatibility of biomaterials, revealed the association of the surface properties on the final stage of the FBR, that is, capsule formation and fibrosis. Studies with confirmed influence of the biomaterial chemistry on capsule formation are rather rare. Ward et al. reported a decreased encapsulation with materials made of polyurethane including silicone and polyethylene oxide moieties as compared to pure polyurethane material [155]. In addition, minimal fibrous reactions were elicited by biomaterials made of gelatin-based hydrogels or hydrogels containing poly (carboxybetaine methacrylate) when compared to for instance poly-2-hydroxyethyl methacrylate. Formation of the thickest capsules were reported with amino and hydroxyl groups on hydrophilic surfaces as compared to other groups [156,157]. On the other hand, carboxyl groups on hydrophobic surfaces were seen to induce the thickest fibrous capsule [158].

Researchers also proposed the covering of the surface with antiinflammatory materials as an approach to reduce capsule formation [113]. Furthermore, studies reported that hyaluronic acid, carboxymethylcellulose, antiadhesive barrier solution, and oxidized regenerated cellulose can decelerate the capsule formation. However, once the surface is degraded and metabolized, normal capsule formation is observed around the implant [159,160].

Andrade reported that, in the case of soft tissues, planar substrates of totally diverse chemical composition (polymers, ceramics, metals) elicit distinct and composition-independent host foreign body responses [161]. It was further evident that the material chemistry is of secondary importance when researchers found out that all classes of materials induced a similar inflammatory reaction [141]. Additionally, if the fact that material composition is the primary mechanism for the foreign body response, a more severe reaction would be expected with rough materials because the material's surface is much larger compared to a planar surface. However, the findings were conflicting. Thus, replacing a planar surface with a microstructured surface for the same material attenuated the foreign body response in experimental as well as clinical studies [162–165].

6.5 Conclusion

The complex interaction at the interface between the implanted biomaterial and surrounding host tissue is key to determining the success of the regeneration process. A meticulous understanding of the mechanisms that elicit the cellular and molecular responses against the implanted biomaterial is a mandate to address the critical issues that affect the success of tissue engineering. Although numerous studies have attempted to elucidate this multiplex and interrelated biomaterial-immune system interplay, a knowledge gap still exists in interpreting the specific processes responsible for attaining optimal regeneration. Recent researches have revealed that biomaterial surface characteristics such as topography, protein adsorption, degradability, mechanical properties, porosity, etc. greatly influence the initial processes (adhesion, activation, and differentiation) and the subsequent foreign body response. As a result of which, designing of biomaterials is gaining much attention and engineers are attempting to manipulate the physical and chemical properties of the biomaterial that can potentially modify the immune system thereby

avoiding an overshooting of the FBR. Thus, the success or failure of clinical outcome depends on the ability of the implanted biomaterial to trigger a desirable immune response. Continuing and in-depth knowledge of the molecular and cellular responses elicited by these tissue-engineered materials is of great significance, paving way for innovative and sophisticated designs in the field of biomaterial science and tissue engineering.

Conflict of interest

The authors declare no conflict of interest.

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Cell–biomaterials interactions: the role of growth factors

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7.1 Introduction

While lucky salamanders carry superhero-like abilities, the regeneration potential after injury appears very limited in most human tissues and organs. Bones have the amazing capacity for self-repair, an uncommon property in adult tissues. Despite this inherent ability, there are a number of clinical situations with critical-size bone defects (with spanning >2 cm), in which complete bone healing fails to occur. Organ/tissue transplantation has traditionally been the first medical choice; emerging thus as a life-saving procedure but consequently creating a significant patients waiting list [1]. Despite progress, limitations still exist including a shortage of donor organs supply, age limit, cost concerns, long time/complex surgical procedures, donor site morbidity, and the continuous need for immuno-suppressant; imposing substantial risks of toxicity and mortality [2].

Tissue engineering emerged as an interdisciplinary field to address organ/tissue transplantation limitations [3]. All started in the early 1970s, at the Boston Children's Hospital, where researchers used chondrocytes and a bony matrix in a unsuccessful attempt to generate a new cartilage [4]. Since then and taking into consideration the possibility to regenerate tissues by loading viable cells onto “smart” engineered scaffolds, this “new era in health care” has successfully expanded and excited researchers worldwide [5]. Many of these explorations have led to encouraging outcomes in in vitro and in vivo models with considerable clinical translation potential [6,7]. From hard to soft, tissue-engineered materials are to meet essential characteristics: (1) provide a platform for cell attachment and

growth; (2) orchestrate cell differentiation or tissue formation through customized bio-physical/chemical cues and/or multiple tissue specific growth factors (GFs) delivery over a therapeutic timespan; (3) be biocompatible with controllable degradation and resorption rate; and (4) matching or narrowing mechanical properties of tissues at the implantation site [8,9].

Aiming to enhance the regeneration process of damaged tissues and organs, modern strategies involve the use of tissue-engineered materials in combination with living cells of interest and/or bioactive molecules (Fig. 7.1).

Therapy based on biomaterials and cells opened new possibilities for the development of innovative medicine. Indeed, biomaterials may improve cell's biological activity either by enhancing their differentiation at the injured site or by enhancing their proregenerative paracrine potential [10–12]. Among cells used in tissue engineering, cell lines are usually

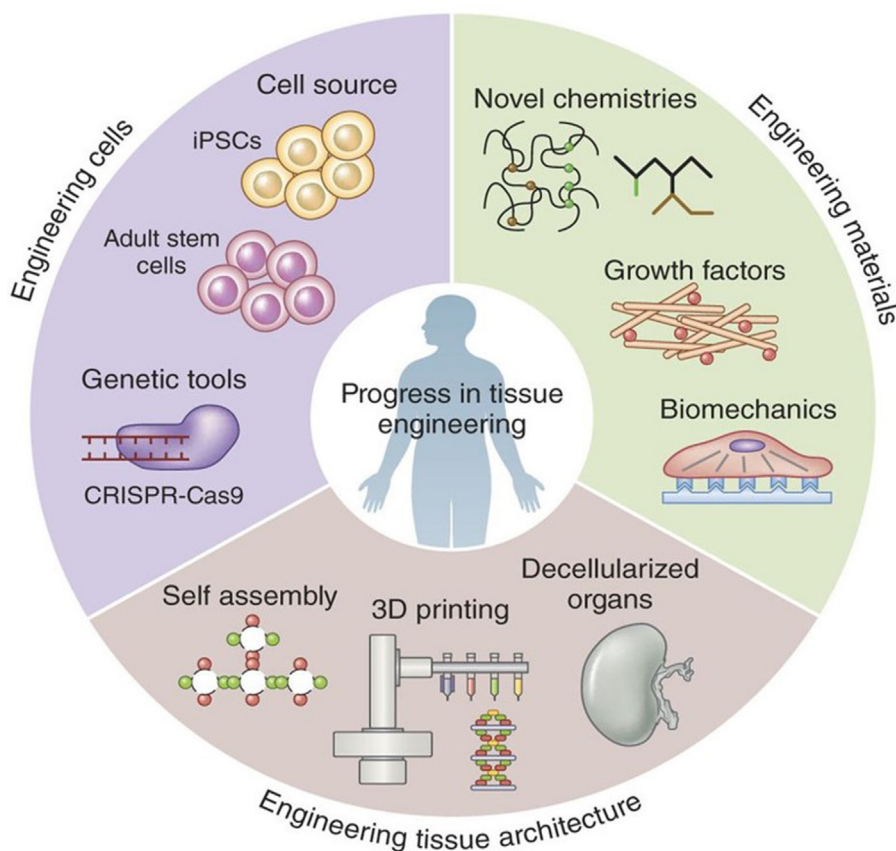


FIGURE 7.1 Summary of tissue engineering progress in the past decade. Strategies for tissue engineering accomplished as a result of improvements and advances in the following areas. Source: Original figure used with permission Khademhosseini A, Langer R. A decade of progress in tissue engineering. *Nat Protoc* 2016;11(10):1775–81. <https://doi.org/10.1038/nprot.2016.123>.

used to test the *in vitro* cytocompatibility of materials however; these cells fail to mimic the *in vivo* behavior of a tissue implant. Autologous or allogenic cells derived from tissue explants, constitute an ideal source of cells more comparable to endogenous progenitors. Depending on the targeted tissue, cells can be painfully and hardly isolated from patients and/or tend to lose their phenotype upon significant *in vitro* expansion [13]. Owing to their capacity to orchestrate, after administration, their differentiation process along with committed resident cells for tissue restoration, pluripotent, and multipotent stem cells caught the scientific and public domain's attention, bringing an understandable high level of hope for new breakthroughs in modern regenerative medicine [14]. Stem cells are extensively investigated as an abundant and appropriate type of cell to be used in tissue engineering due to their ability to differentiate into multiple lineages and to secrete a wide spectrum of molecules with immunomodulatory, antiinflammatory, proangiogenic, proliferative, or chemoattractive capacities [15].

Besides an appropriate scaffold and cell source, signaling molecules or GFs represent an attractive tool and a key factor in tissue engineering and regeneration [16,17]. The use of GFs, revealed a “good face” with excellent clinical therapeutic potential but also an “ugly face” due to safety and cost issues along with limitations concerning their delivery and the resulting therapeutic potency [16,18]. Early attempts to deliver systematically GFs *in vivo* revealed low stability, short effective half-life, and/or rapid degradation (e.g., enzymes, pH, and temperature), and poor pharmacokinetics requiring supra-physiological doses leading to dosage-related adverse effects [18,19]. In light of these limitations and keeping in mind the ultimate goal of mimicking the extracellular matrix (ECM) to locally deliver GFs, conventional carrier materials [i.e., collagen (COL), fibrin-based sponges] have been made, but showed weak capacity to control GFs delivery to reach therapeutic effects [8,20,21]. Hence, more sophisticated bioinspired materials have been developed in a manner to reduce GFs doses and to ensure an active biological conformation and a local concentration/concentration gradient within and around the implanted material. Several papers/reviews in the broader field of materials-based GFs delivery have been recently discussed and published [22–24]; interested readers are invited to refer to these references. Owing to their regenerating potential in various tissue engineering applications, the present chapter discusses and describes the role of soluble/immobilized GFs in guiding, through extra and intracellular mechanisms, the cell/tissue response to engineered materials.

7.2 What are growth factors?

It all began with the question: “How does an egg turn into a chicken or a frog or a person?” and ended, in the beginning of 1950s, with the discovery of two soluble and diffusible factors namely, “nerve and epidermal GFs (EGF),” opening thus a new concept in the study of normal/abnormal development and differentiation of organs/tissues [25]. GFs are soluble signaling molecules secreted by cells and possess the capacity to mediate, upon binding to specific receptors of the same (autocrine) or neighboring (paracrine) cell, a large spectrum of events including cytoskeleton reorganization, changes in ion flux and metabolism, gene expression, and finally protein synthesis [26]. GFs are key actors in the

tissue engineering of various tissues due to their effect on cell response and behavior such as migration, differentiation, and survival. The latter is not only determined by the nature of GFs and the ability to diffuse through their carrier (i.e., ECM, hydrogels, and nanoparticles) but also by the cell type and/or binding receptor and the resulting intracellular signal transduction [18]. Inspired from bone tissue applications, we will overview these aspects and their effect on the in vitro as well as in vivo cell behavior and on the cell/material interactions.

7.3 Growth factors in bone tissue engineering

A wide range of GFs families are found to be involved in the regeneration of bone; we can cite the bone morphogenetic proteins (BMPs), transforming GF- β (TGF- β), platelet-derived GF (PDGF), fibroblast GF (FGF), insulin-like GF (IGF), and vascular endothelial GF (VEGF). A special consideration will be given for the BMPs family as BMP-2 and BMP-7 have been approved by the Food and Drug Administration to be used as devices for bone regeneration [8].

7.4 Bone morphogenetic proteins

The ability of autologous demineralized and lyophilized bone matrix to induce ectopic bone formation in rabbit led to the discovery of BMPs as a potent osteoinductive GF, belonging to the superfamily of TGF- β , to be used in bone tissue engineering [27]. Indeed, upon bone fracture, BMPs secreted by stem cells arriving to the site are likely to help recruiting and then differentiating progenitor cells into bone forming cells, reducing thus the duration of fracture healing. Secreted BMPs, interacting directly with cells or “stored to act later” in the newly formed ECM, at different temporal scales play a key role in several steps of fracture healing including stimulating angiogenesis, inflammation, cartilage formation and resorption, primary and second bone formation and remodeling [28]. On cells, BMPs act by binding to serine/threonine kinase type I receptors namely BMPR-IA, BMPR-IB, and ActR-IA (ALK-2); and type II receptors namely BMPR-II, ActR-II, and ActR-IIB [29]. BMPs and cell surface receptors interaction could result in the phosphorylation of Smad 1/5/8 and their association with Smad 4 leading to the translocation of the complex into the nucleus. This is thought to induce the transcription of genes mediating cell differentiation such as *Runx2* and *Osterix* and other genes like *alkaline phosphatase* (ALP), *osteocalcin* (OCN), *COL-I*, and *bone sialoprotein* [30]. The Wnt signaling pathway plays a key role in the bone tissue remodeling, this pathway is also activated upon BMPs/receptor interaction leading to the activation of specific genes like *c-Jun* involved in the early differentiation of bone cells [31,32]. The activation of a mitogen-activated protein kinase (MAPK) pathway, playing an important role in cell commitment into osteoblastic lineage, is also mediated by BMPs proteins [33]. Among identified BMPs, BMP-2 and BMP-7 have drawn attention for their successful osteoinduction in preclinical studies and their access to clinical trials to treat multiple bone disorders [34]. Delivering these GFs in clinic is, however, limited to absorbable COL-I sponge, yet BMPs present poor affinity for COL and are rapidly cleared

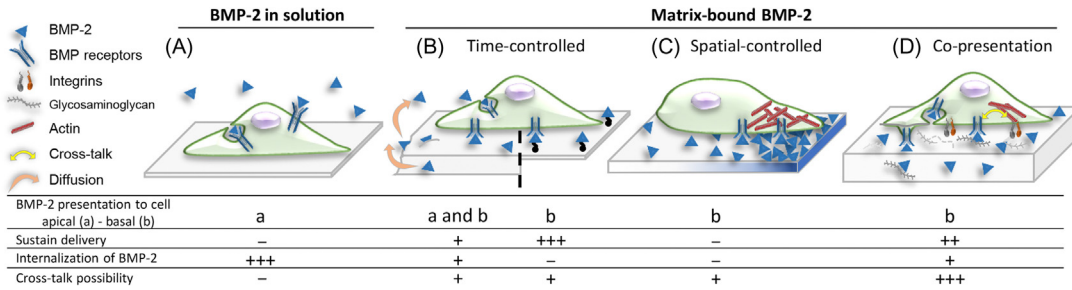


FIGURE 7.2 Different modes of presenting BMP-2 at the interface material/cell. (A) Soluble BMP-2 within the cell culture media. (B) Controlled immobilization and/or release of the protein from the surface: left: BMP-2 entrapped by electrostatic interactions or right: BMP-2 chemically bound to the material. (C) Spatial controlled release due to surface chemical modifications and BMP-2 patterning. (D) BMP-2 is presented together with ECM components. BMP, Bone morphogenetic protein; ECM, extracellular matrix. Source: Original figure used with permission Migliorini E, Valat A, Picart C, Cavalcanti-Adam EA. Tuning cellular responses to BMP-2 with material surfaces. *Cytokine Growth Factor Rev* 2016;27:43–54. <https://doi.org/10.1016/j.cytogfr.2015.11.008> [48].

(<14 days) in vivo [20]. Emerging approaches have been gathered on optimizing GFs delivery with the aim of minimizing the needed dose and extending the release (at least 3 weeks) and the bioactivity over longer timescales [8]. The latter constitutes an essential parameter, especially when it comes to treating large bone defects, where the stabilization of GFs on the surface of materials/coating seems to be necessary. Delivering BMPs was achieved using (1) natural polymers such as COL [35], fibrin/fibronectin [36], chitosan, and hyaluronic acid [37]; (2) inorganic materials such as hydroxyapatite (HaP) [38], tricalcium phosphate (TCP) [39]; (3) synthetic polymers like poly lactic acid [40]; and (4) composite organic/inorganic materials [41,42]. Among these materials, natural polymers and composite materials were reported to improve the bioactivity of GFs [43,44]. Chitosan constitute a plentiful and a valuable antibacterial, biocompatible and biodegradable natural polymer. In addition, it possesses the versatility advantage to be designed into any size or shape (i.e., nanoparticles, microspheres, nanofibers, porous scaffolds, gel, and films) [37,45]. BMP-2 has been directly immobilized on a guided bone-regenerative membrane surface made of chitosan nanofibers, providing a bioactive surface (up to 4 weeks) that increased osteoblastic cells attachment, ALP activity, as well as calcium deposition [46]. Studying the effect of BMP-2 sustained release from an injectable chitosan gel highlighted a cell and species dependent effect, with respectively an enhanced ALP activity and mineralization for preosteoblast mouse stromal (W-20-17) and human embryonic palatal mesenchymal cells [47]. The bioactivity of BMPs is also affected by the scaffold binding and/or loading techniques and/or the mode of presentation of these GFs at the interface with cells (Fig. 7.2).

BMP-2 was immobilized onto poly(L-lactide)-*co*-(ϵ -caprolactone) (PLCL) scaffolds using four techniques: (1) physisorbed on unmodified scaffold; (2) physisorbed onto scaffold modified with nanodiamond particles; (3) covalently linked onto nanodiamond particles used to modify the scaffold; or (4) encapsulated in microspheres distributed on the scaffold. Despite the close similarity of in vivo results and the potential use of these

functionalizing methods in clinic, *in vitro* results highlighted significant differences in the osteogenic effect on human mesenchymal stem cells (hMSCs) [49]. Others preferred a chemical functionalization-free approach to release low doses of BMP-2 from a COL-I/ HaP scaffold in order to achieve *in vitro* and *in vivo* bone regeneration using 30 times less BMP-2 than the clinical gold standard INFUSE [41]. Although not fairly addressed in the literature, strategies using bioresponsive degradable scaffolds, or cell-triggered degradation approaches, could present a way to achieve temporal stability and sustained release of osteoinductive GF from scaffold. Both degradation rate and GFs liberation is likely to depend on cell-secreted protease during local tissue remodeling, leading to a physiologically more adapted regeneration [50] (Fig. 7.3).

The ECM orchestrates the activity of GFs, regulating their local concentration, bioavailability, and bioactivity and thus constituting a key regulator for various cell behaviors.

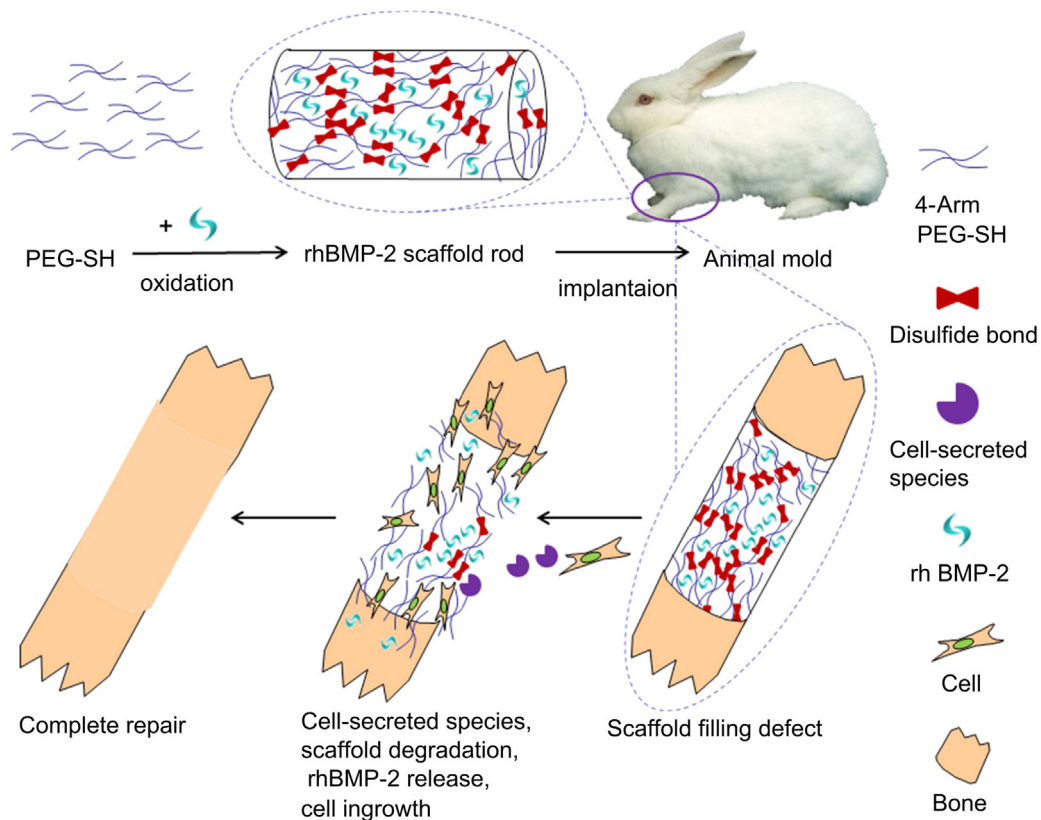


FIGURE 7.3 *Responsive-degradable scaffold for bone repairing applications.* Upon implantation into radius segmental defect and cell repair recruitment, disentanglement of disulfide-bridges is likely to occur following cell-secreted protease secretion, leading to the liberation of rh-BMP-2 and a physiologically more adapted bone regeneration. BMP, Bone morphogenetic protein. Source: Original figure used with permission Yang F, Wang J, Hou J, Guo H, Liu C. Bone regeneration using cell-mediated responsive degradable PEG-based scaffolds incorporating with rhBMP-2. *Biomaterials* 2013;34(5):1514–28. <https://doi.org/10.1016/j.biomaterials.2012.10.058>.

The dynamic GFs/ECM interaction is crucial for tissue regeneration and can be expressed directly by binding to or by releasing GFs or indirectly through the ECM/cell integrin-mediated interactions [51,52]. Materials mimicking GF interactions with proteins derived from the ECM (i.e., fibronectin) may provide tools of interest for more localized and efficient GFs low-dose delivery [36]. It is well known that fibronectin adsorbs onto polymers in globular conformation, bioactive polymers like poly(ethyl acrylate) are able to induce fibronectin organization into nanonetworks exposing both integrin-binding (III9–10) and GF-binding (III12–14) domains. Such mechanism allows a synergistic interaction at the integrin/GF receptor level and stably presents GFs such as BMP-2 [36,53] or BMP-7 [54], at effective and ultralow doses (~ 300 -folds lower GF dose than with COL sponge delivery). Importantly, these studies revealed the ability of such approach to be more potent in terms of osteoinduction [36,53]. Several works have shown that enhanced focal adhesion and BMP-2 proteins or peptides could contribute to cell osteogenic differentiation promotion [53,55,56]. Indeed, BMP-2 receptors were found to be overlapped with α_v/β_1 integrin subunits at focal adhesion points; improving thus, the interaction between BMP-2 receptors and their ligands sequestered within the ECM. BMP-2 can also increase the level of α_v/β_1 integrins on the cell's surface, favoring their adhesion and amplifying the synergistic interaction at the integrin/GF receptor level [57]. In some cases, integrins can negatively regulate GF receptor signaling through their ability to induce a phosphatase activation and recruitment to inhibit the signaling of GF receptors [52,58].

7.5 Transforming growth factor β s

In the late 1970s, culturing virus transformed rat kidney fibroblasts revealed the presence of a new polypeptide called sarcoma GF was found lately to be composed of two GFs [59]. TGF- α , structurally similar to EGF and a ligand for EGF-receptors, involved in the growth/proliferation of a broad range of cell types and TGF- β with multiple regulatory properties including growth, development, ECM deposition, tissue homeostasis, and regulation of the immune system [60]. The latter, expressed under three known isoforms (TGF- β_1 , TGF- β_2 , and TGF- β_3), acts physiologically as an actor in the tissue regeneration/remodeling process through the recruitment of stem/progenitor cells, but pathologically as a mediator for a broad range of pathologies such as cancer, cardiovascular pathology, fibrosis, and congenital diseases [61]. Unlike most of the GFs, TGF- β is secreted as part of a latent complex that is stored in the ECM for activation (i.e., by matrix metalloproteinases, integrins, reactive oxygen species, and by the acidic pH generated by osteoclasts during bone resorption) and for action at a later time point [60]. Notice that these GFs present a short half-life once in vivo, with about 2–3 minutes for the active form of TGF- β_1 and >100 minutes for the latent [62]. As for TGF- β ligands, three isoforms of TGF- β receptors acting with different affinities were reported. The first two are transmembrane serine/theronine kinases while the third has no kinase activity but has the capacity to bind the three TGF- β ligands with high affinity and is known to promote binding of TGF- β_2 to TGF- β R2. Such binding is likely to activate canonical (i.e., SMAD proteins phosphorylation and transcription of TGF- β dependent genes) or noncanonical signaling pathways (MAPKs, ERK, P38, JNK, phosphatidylinositol 3 kinase (PI3K)/PKB, or ROCK) and to

regulate the activation of other signaling pathways [60,63,64]. The role of TGF- β , despite controversial results, in bone fracture healing and bone tissue engineering has been extensively studied and demonstrated [60,65,66]. TGF- β s have been applied to bone-based materials to modulate the local delivery and signaling in favor of bone formation, recruitment of osteoprogenitors to the implant site, their adhesion, proliferation, and differentiation along with the synthesis of bone ECM and its mineralization [64,67]. TGF- β is likely to protect ECM from degradation by increasing the synthesis of protease inhibitors and/or by inhibiting the secretion of protease [68]. Moreover, it was shown that localized TGF- β 1 delivery is able to modulate the immune response to biomaterial implants (Fig. 7.4) and enhance cell function in cell-based therapies. This hypothesis was investigated using poly (lactide-*co*-glycolide) (PLGA) scaffolds that supported islet transplantation into diabetic mice. The scaffold maintained TGF- β 1 bioactivity and provided short-term release along with a decreased leukocyte infiltration and inflammatory cytokine production within the implant, delaying thus the graft rejection [69]. Loading HaP microspheres (150–250 μ m in diameter), created using a glass conversion process, with TGF- β 1 (5 μ g/defect) resulted in a significant increase in bone regeneration up to 6–12 weeks postimplantation in rat calvarial defects [70]. In a coral-derived calcium carbonate-based macroporous bioreactors with limited conversion to HaP, loaded TGF- β 3 (125 μ g/bioreactors) was able to modulate BMP-2 temporal/spatial expression patterns, affecting thus the bone cellular differentiation and proliferation pathways [71]. One could also say that TGF- β 1 enhances stem cells' bone lineage commitment by regulating the morphology of the actin cytoskeleton and focal adhesion into the material via the TGF/MAPK signaling pathways [72].

Natural scaffolds including COL [73], bulk chitosan [68] or modified (e.g., chitosan-silk-fibroin, chitosan-glass ionomer cement) [74,75] combined to TGF- β 3 were successfully developed to generate bone cell/tissue. Scaffold based on COL-I/glycosaminoglycan supports in vitro clonal human osteoblast cell differentiation and tissue mineralization.

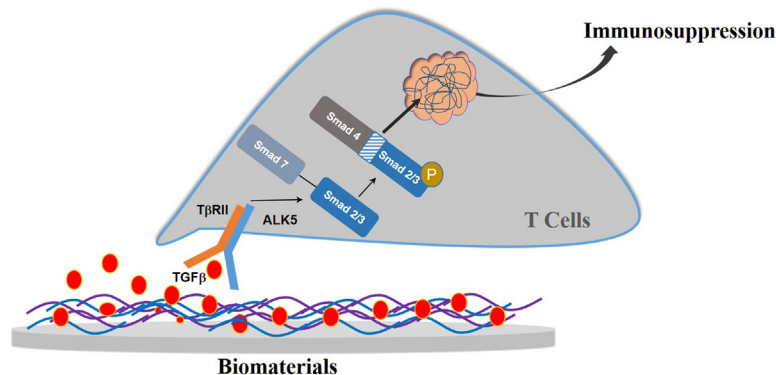


FIGURE 7.4 TGF- β -based immunosuppression in the implant microenvironment. Biomaterial-based delivery of exogenous TGF- β initiates the transcriptional inhibition of inflammatory cytokines, recruitment, and proliferation of various resident macrophages and T-cells to attenuate host immune responses and inflammation. TGF- β , Transforming growth factor β s. Source: Adapted with permission Kwak E-A, Lee NY. Synergetic roles of TGF- β signaling in tissue engineering. *Cytokine* 2019;115:60–3. <https://doi.org/10.1016/j.cyto.2018.12.010>.

Expression of *ALP*, *COL-I*, *osteonectin*, *bone sialoprotein*, *biglycan*, and *OCN* after 35 days highlighted the benefit of introducing an initial high treatment of TGF- β 1 (10 ng/mL) followed by a low continuous treatment (0.2 ng/mL) [73]. In the early 1990s, Bentz et al. claimed that a method for treating bone and cartilage formation in individuals comprises the administration of BMP-2, BMP-3, and TGF- β 3 together in a tissue-growth-inducing amount, and a pharmaceutically acceptable carrier or excipient (US patent no. 5.393.739) [76]. Indeed, TGF- β /BMPs have key roles in bone formation during mammalian development and exhibit versatile functions in the body [77]. Loaded within alginate hydrogels or gelatin films sandwiched between PLCL matrices, TGF- β 3/BMP-2 were found to be superior in bone formation in comparison to empty and GF-single loaded scaffolds, exhibiting autograft implants-like performance [78,79]. Similar results were reported in vitro/ in vivo with hydrogel systems based on visible light-cured glycol chitosan and bilayered alginate/PLGA scaffold combined with TGF- β 1/BMP-2 [80,81]. Combined with a non-BMP, GF was also reported where freeze-dried bone allografts supplemented with PDGF-BB and TGF- β 1 resulted in a significant upregulation of MG-63 osteoblast-like cells bone related markers and in a higher amount of mineralized nodules compared with unsupplemented allograft [82].

7.6 Platelet-derived growth factors

Bone fracture healing is a complex and well-orchestrated regenerative process involving numerous signaling pathways and cell types. Immune cells such as platelets are recruited to the fracture site and activated, leading to the release of GFs and cytokines essential to control mesenchymal cells recruitment, activation, proliferation, and differentiation [83]. Among released GFs, the PDGF, a chemotactic protein discovered and characterized in the late 1970s [84–86], once released from platelets it binds to specific cell surface receptors promoting rapid cell migration and proliferation within the bone fracture site [87]. PDGF has various isoforms (AA, AB, BB, CC, and DD) and binds to cells through two distinct receptors (α and β) with tyrosine kinase activity [88]. PDGF-AA mainly activates PDGFR α while PDGF-BB activates PDGFR β . This would likely activate main signaling pathways such as MAPKs, PI3K, Stat3, and the Rho/Rac cascades, which control cell proliferation, migration, and survival [89,90]. PDGFRs are known to be expressed throughout the fracture healing process in various cell types (mesenchymal, osteoblasts). Lynch and colleagues were the first to highlight the role of PDGF in promoting the in vivo bone regeneration [91]. Combined with composite fibers [92], mineralized-COL matrix [93], bioactive COL membrane [94], calcium phosphate/alginate scaffold [95] or alloplastic bone matrices [96], PDGF revealed an early cell osteogenic potential. PDGF-BB was successfully incorporated into electrospun poly-lactide/HaP/COL composite fibers or bioactive COL membrane. With a sustained release ($\sim$ 3 weeks), PDGF-BB was able to increase human bone marrow (BM)-MSCs, or MC3T3 cell attachment/proliferation and the expression of fibronectin, intracellular adhesion molecule; and COL-I. PDGF-BB also had a positive effect on cell differentiation through an upregulation of specific bone markers expression (genes and proteins) including *Runx2*, *COL-I*, *Osteonectin*, *Bone gamma-carboxyglutamic acid-containing protein*, *Osteopontin*, and *BMP-2* (Fig. 7.5) [92,94].

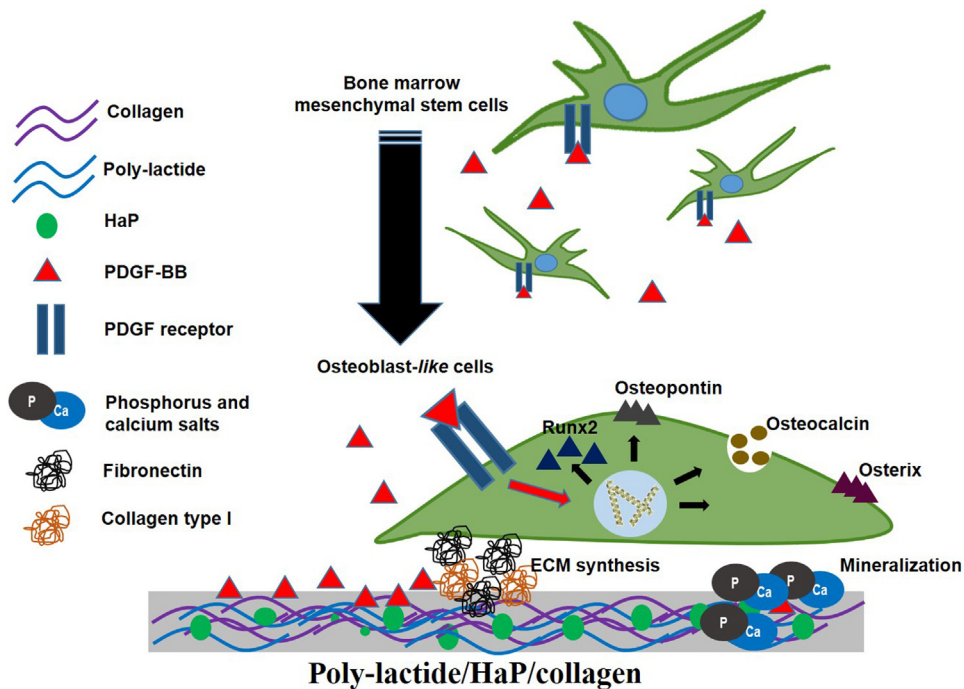


FIGURE 7.5 Effect of PDGF and PLLA/Col/HA interaction on the stem cells bone-like differentiation. PDGF-BB acts synergistically with poly-lactide/HaP/collagen to enhance BM-MSCs osteogenic differentiation potential through an increase in cell attachment to biomaterials, the synthesis of a new ECM and an increase in the expression of bone cell specific markers (genes and protein) [92]. BM, Bone marrow; ECM, extracellular matrix; HaP, hydroxy-apatite; MSC, mesenchymal stem cell; PDGF, platelet-derived growth factors.

The recruitment of MSCs is a vital step in the bone healing process. Functionalizing bone-based materials with chemotactic factors constitutes an important approach in the tissue-engineering field. In a MSC chemotaxis assay, and compared to PDGF-AB, BMP-2, and a mixture of chemokines SDF-1a, CXCL16, MIP-1a, MIP-1b, and RANTES, PDGF-BB was found to be the most effective in stimulating MSCs migration [97] (Fig. 7.6). PDGF and BMP-2 were sequentially released from a hybrid calcium phosphate/alginate scaffold with a desired 3-day overlap in PDGF to BMP-2 delivery. Such delivery mode encouraged both cellular hMSCs infiltration and bone differentiation when compared to mono-loaded scaffolds (BMP-2 or PDGF) [95].

Despite recent controversial reports [98], the ability of PDGF to promote the *in vivo* bone regeneration has been highlighted in various animal models [19]. Chitosan or acellular COL sponge, containing 0.03% PDGF, enhances bone formation beyond the skeletal envelope in rat calvaria compared to control groups after 4 weeks [99,100]. Adapting the same animal model, Shah and colleagues reported an approach for bone repair using a polyelectrolyte multilayer coating carrying as little as 200 ng of BMP-2/PDGF-BB that were eluted over readily adapted time scales to induce rapid bone repair (~2 weeks). Based on electrostatic interactions between the polymer multilayers and GFs alone, they

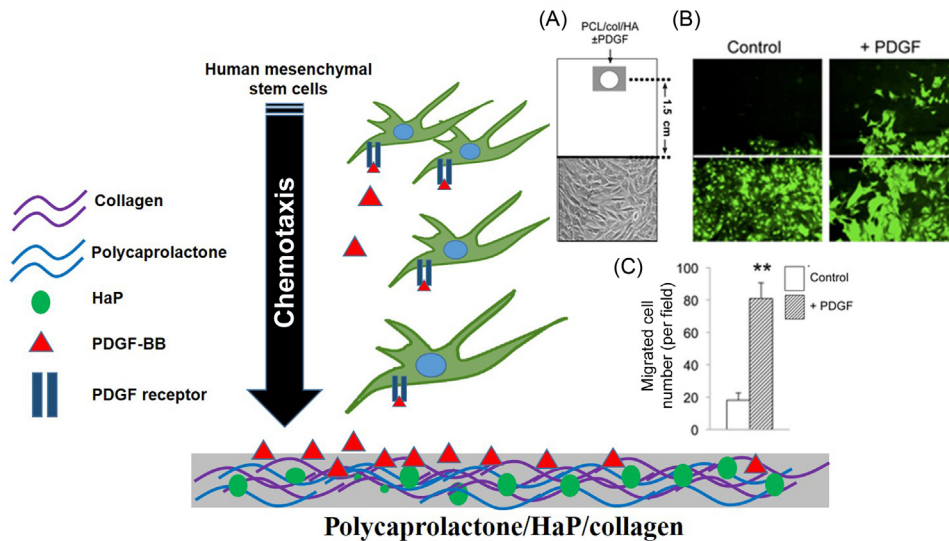


FIGURE 7.6 PDGF-BB as a chemotactic factor for MSCs on bone-mimetic electrospun scaffolds. Representative schema showing the effect of incorporating native bone molecules such as collagen I and HaP, into electrospun scaffolds on MSCs attraction and adhesion. (A) Representative schema of the new type of migration assay used to demonstrate the ability of PDGF-BB-loaded scaffold to attract MSCs, (B) fluorescence footage highlighting the migration of cells towards scaffold, and (C) quantification of the migrated MSCs due to released PDGF-BB [97]. HaP, Hydroxyapatite; MSC, mesenchymal stem cell; PDGF, platelet-derived growth factors.

were able to sustain GFs mitogenic and osteogenic signals in a controlled manner to direct endogenous cell function [101]. Delivering PDGF-BB via HaP/PLGA microspheres/Pluronic scaffolds or via a molded poly/TCP membrane revealed an improvement in bone regeneration in the rabbit model with a new bone formation at 4 weeks [102,103]. Combining rhPDGF-BB with a deproteinized block of bovine bone improved outcomes of vertical alveolar ridge defect in a standardized dog model with quite an amount of new bone formation without the use of a specific membrane [104]. The addition of PDGF-BB to a COL matrix (Mucograft) applied on a titanium mesh is likely to favor bone tissue healing, reduce mesh exposure, and protect the grafted bone in a pig jaw defect model [105].

7.7 Fibroblast growth factors

In their work in the early 1930s, Trowell and Willmer reported that embryo and brain extracts promote the growth of chicken periosteal fibroblast [106]. Thirty years later, FGFs including FGF-1 and FGF-2 were isolated from the brain and pituitary as mitogens for cultured fibroblasts and since, at least 22 distinct FGFs have been identified or isolated [107,108]. Among these molecules, paracrine and endocrine FGFs act via ligand-dependent FGF receptor tyrosine kinase (FGFR1–4) while, intracrine FGFs act independently of FGFRs [109,110]. They also present a high affinity for heparan sulfate proteoglycans and

can interact with heparin-like glycosaminoglycans of the ECM, where they are normally, upon cell secretion, stored and protected against degradation until further use. In addition to their mitogenic effects, activation of FGFRs triggers distinct pathways [e.g., Ras/MAPK, PI3K/Akt, phospholipase C γ (PLC γ) and protein kinase C pathways], leading to the modification of cell behavior including cell survival, migration, differentiation, and apoptosis [111–113]. Multiple FGFs and FGFRs are dynamically expressed during fracture healing and each of the FGFs/FGFRs has its own unique spatiotemporal expression patterns and/or roles during bone regeneration [114]. The latter was already checked in a mouse long bone fracture model using gene quantitative assays. FGF-1/2/5 and FGFR1/R2 were found to be significantly upregulated at day one postfracture. The 4–9 day postfracture or chondrogenesis stage highlighted an upregulation in FGFR3 and FGF-16/18 expressions while maintaining FGF-1/2/5 high levels. Finally, resolving chondrogenesis and ongoing osteogenesis at day 14 resulted in an upregulation of FGFR1/R2/R3 and FGF-1/9/16/17/18 [115]. In addition, it was reported that altering FGF signaling pathway is likely to contribute to impaired osteoblasts bone formation capacity and to increased BM fat accumulation both of which are characteristics of aged bone [116]. Thus, immobilizing FGFs on biomaterials and/or their local delivery represents a promising approach to favor osteoblastogenesis and to restore bone defects. Keeping in my mind that vision, silicon-substituted HaP were used as materials to immobilize bioactive FGF-1 and FGF-2 and the analysis of their specific signal transduction pathways was carried out on both osteoblasts (Saos-2) and preosteoblasts (MC3T3-E1). It was shown that these immobilized GFs provided the right signals to cells stimulating crucial intracellular mechanisms of osteoblast and preosteoblasts adhesion, proliferation, and differentiation through PLC γ and MAPK/ERK pathways [117,118]. One could report that FGFs like other GFs are likely to play the role of connector between cells and material/matrix and this connection is more effective if FGFs are located between cell and material/matrix instead of the continuous injection into the culture media. Others went to estimate the optimal dose of covalently immobilized FGF-2, onto biphasic calcium phosphate (BCP), resulting in the best osteogenic differentiation of hMSCs. This type of immobilization is suitable to create strong bonds between FGF-2 low amounts and the substrate and especially to avoid FGF-2 overdose regardless of the type of biomaterial. Results of biological assays revealed that high concentrations of FGF-2 (400 ng) reduced the initial attachment of hMSCs to BCP while weak concentrations (50–100 ng) of FGF-2 were enough to promote the ALP activity of hMSCs [119] (Fig. 7.7). Such effect on hMSCs is reported to be mediated through ERK-induced transcriptional coactivator with PDZ-binding motif (TAZ) expression, where FGF-2 is likely to increase nuclear localization of TAZ and, thus, improving the interaction of TAZ/Runx2 and activating Runx2-mediated gene transcription [121]. FGF-2 is known to increase osteoprogenitor cells sensitivity to BMP-2 by upregulating receptor expression and BMP-2 levels. Core/shell microspheres with poly L-lactide (core)/PLGA (shell) and biomimetic calcium phosphate covered with a poly-Lysine/PLGA multilayer film were used to encapsulate these two GFs [122,123]. The effects of different release patterns (parallel or sequential manners) of FGF-2 and BMP-2 from the core/shell microspheres on the osteogenic differentiation of low-population density hMSCs were investigated.

In vitro experiments suggested that induction of hMSCs osteogenic differentiation by the sequential delivery of FGF-2 (proliferation) followed by BMP-2 (osteo-differentiation) is the

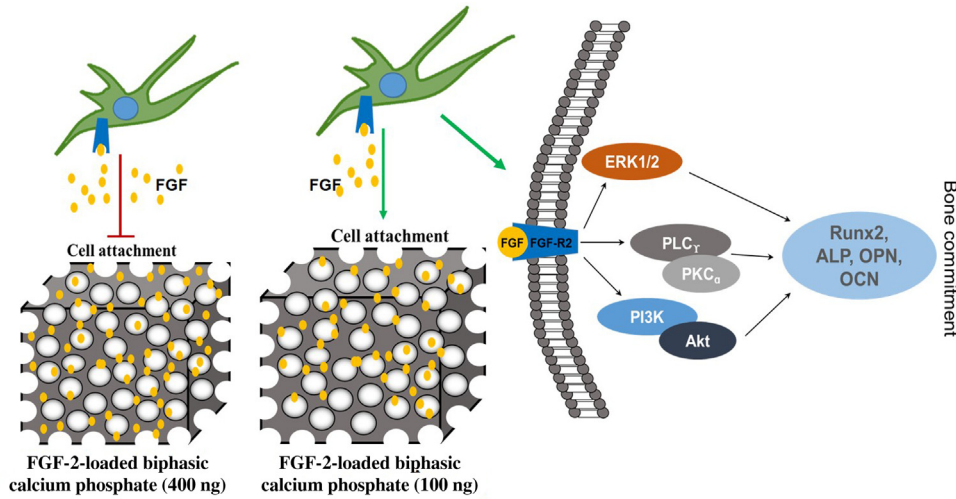


FIGURE 7.7 Effect of immobilized FGF-2 dose on biphasic calcium phosphate bone substitute on enhanced cellular activity. Representative schema showing the effect of grafted FGF-2 dose on cell attachment and the resulting cellular mechanism [119,120]. FGF, Fibroblast growth factors.

ideal condition when compared to the parallel release and that delivering BMP-2 then FGF-2 had drastic outcomes on cells [123]. Following the same hypothesis, Kang et al. reported the use of a bone scaffold where FGF-18, preloaded within mesoporous bioactive glass nanospheres, was incorporated within an FGF2-loaded core/shell electrospun polymeric fiber where FGF-2 is released initially to stimulate cellular proliferation and possible angiogenesis, while FGF-18 is released more slowly to promote osteogenesis [124] (Fig. 7.8). FGF-7 delivered through acellular COL membrane was also reported to enhance bone formation by inducing SDF-1/CXCR4 expression and stimulating thereby the migration of stem-like cells into the bone implant at the defect region. Such effect is also associated with an upregulation of specific osteogenic marker genes through the activation of JNK and ERK signaling pathways.

The potential use of FGFs in small and large animal models has been widely evaluated [19,125,126]. In a mouse cranial bone defect model, FGF-18 loaded chitin-PLGA/CaSO₄ gel showed a sustained release of FGF-18 and an early and almost complete bone healing in comparison with controls. The latter was due in part to the presence of CaSO₄ but also to FGF-18, increasing thus the endogenous synthesis of BMP-2 and expression [127]. FGF-2 (0.1% or 0.3% concentration) released via COL sponge promotes, in a concentration-dependent manner, high blood vessel and bone volume formation at 28 days in rat calvarium subcritical and critical defects [128,129]. Recently, Lee et al. applied different FGF-2 concentrations to BCP bone graft site for guided bone regeneration in rabbit calvarial defects. Formation of new bone and a decrease in the amount of residual graft material was noticed in all experimental groups compared to empty membrane, however at 12 weeks, FGF at 1 and 0.5 mg/mL showed larger areas of new bone compared to 0.1 mg/mL at 12 weeks [130]. Nano- β -TCP/COL scaffold loaded with FGF-2 significantly promoted the repair of periodontal tissues, including bone and cementum-like tissue, while suppressing the gingival recession in a dog one-wall periodontal defect model [131].

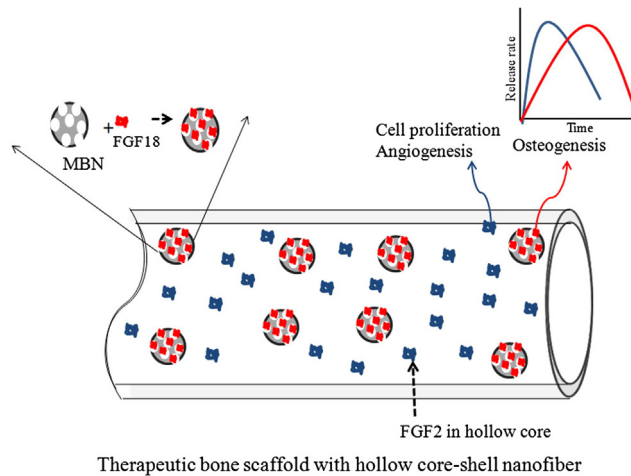


FIGURE 7.8 Effect of sequentially delivery of dual FGF-18 and FGF-2 on human MSCs. Schematic design of a therapeutic bone scaffold containing mesoporous bioactive glass nanospheres preloaded with FGF-18 incorporated within an FGF-2-loaded core–shell electrospun polymeric fiber. FGF-2 is released initially to stimulate cellular mitosis and possible angiogenesis, while FGF-18 is released more slowly to promote osteogenesis. FGF, Fibroblast growth factors; MSC, mesenchymal stem cell. Source: Original figure used with permission Kang MS, Kim J-H, Singh RK, Jang J-H, Kim H-W. Therapeutic-designed electrospun bone scaffolds: mesoporous bioactive nanocarriers in hollow fiber composites to sequentially deliver dual growth factors. *Acta Biomater* 2015;16:103–16. <https://doi.org/10.1016/j.actbio.2014.12.028>.

7.8 Insulin-like growth factors

More than 60 years ago, an experience carried out by Salmon and Daughaday led to think that “growth hormone does not support growth process but rather induces the formation of factors that mediate the message of growth hormone.” Nowadays this theory is still valid and these factors, formerly called “sulfation factors” for their ability to stimulate ³⁵sulfate incorporation into rat cartilage, are now called somatomedine or IGFs based on their structural and functional similarity with insulin [132,133]. IGFs, referred to as IGF-1 and IGF-2, bind to receptors IGF-1R and IGF-2R belonging to the tyrosine kinase receptor family and are produced from bone ECM, osteoblasts, chondrocytes, and the liver [134]. IGFs play a key role in fracture healing by promoting bone matrix formation (production of COL-I and noncollagenous proteins) [135]. IGF-1, the most potent and abundant in the bone matrix, is highly expressed in human fracture callus at the stage of cartilage and bone formation and is released upon fractured bone matrix osteoclasts resorption, acting as a mitogenic factor stimulating the growth and differentiation of bone progenitor cells [136,137]. Like mentioned before, immobilizing GFs on biomaterials is likely to extend the activation of cellular signal transduction systems and favoring thereby cell differentiation, migration, proliferation, etc. Indeed, immobilizing IGF-1 on PLGA/HaP microcarriers, via polydopamine coating, was shown to improve mouse adipose-derived stem cells attachment/proliferation and more importantly to enhance ALP activity and expression of osteogenesis-related genes after seven days [138] (Fig. 7.9).

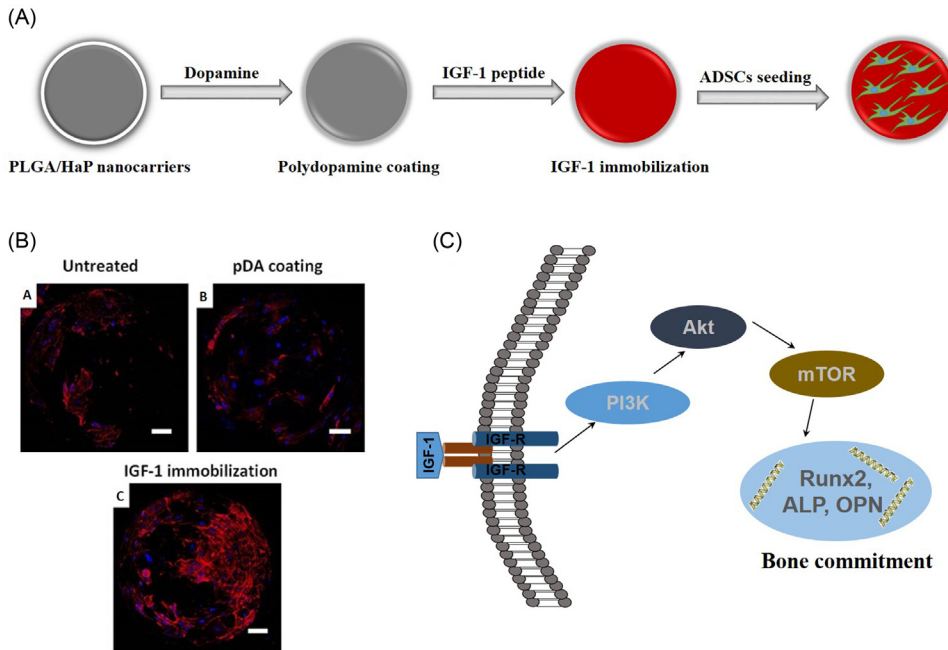


FIGURE 7.9 Effect of IGF-1 immobilization on ADSCs proliferation and differentiation. (A) Schematic design of the study showing the immobilization of IGF-1 on PLGA/HaP nanocarriers via PDA. (B) The positive effect of IGF-1 immobilization on ADSCs proliferation on the nanocarriers compared to untreated or PDA-coated nanocarriers. (C) IGF-1/IGF-R possible signaling pathway [139] in stem cells bone differentiation showing an upregulation of bone gene markers through an interaction between PI3K, AKT, and mTOR. ADSC, Adipose derived stem cells; IGF, insulin-like growth factors; PDA, polydopamine; PLGA/HaP, poly(lactide-co-glycolide)/hydroxyapatite. Source: Adapted with permission Gao T, Zhang N, Wang Z, Wang Y, Liu Y, Ito Y, et al. Biodegradable microcarriers of poly(lactide-co-glycolide) and nano-hydroxyapatite decorated with IGF-1 via polydopamine coating for enhancing cell proliferation and osteogenic differentiation. *Macromol Biosci* 2015;15(8):1070–80. <https://doi.org/10.1002/mabi.201500069>.

Many studies have shown that IGF-1 is able to enhance the in vitro osteogenic potential of BMPs including BMP-2, -6, -7, and -9 [140–144]. Such effect is likely to depend on the temporally varying or sequential delivery of these GFs [136,145]. Chitosan gel/gelatin microspheres or two layered cross-linked gelatin coatings were applied to sequentially release BMP-2 and IGF-1 to enhance BM and W-20-17 cells' osteoblast differentiation in vitro. Providing an initial release of BMP-2 followed by a slow and sustained release of IGF-1 or BMP-2/IGF-1 (from 5 to 14 days), such a combinational delivery system, elicited great levels of osteoblastic activity in term of cells ALP activity and mineral deposition [136,145]. While in vitro studies were focused on the duo IGF/BMP-2, in vivo studies were more focused on the combination with TGF- β and their potential effect on bone formation [146–149]. The effect of different GFs (IGF-1, TGF- β 1, and BMP-2) locally released from coated intramedullary implants on rats fracture healing was investigated. All conditions stimulated the fracture healing however, the local application of combined IGF-1 and TGF- β 1 had the most stimulating effect on fracture healing, followed by the effect of BMP-2, IGF-1, and TGF- β 1 alone [146]. Similarly, bone formation was enhanced

by incorporating TGF- β 1/IGF-1 in the poly(D,L-lactide) (PDLLA) coating of titanium wires implanted in rats and PDLLA membranes in a sheep model of delayed callus formation, showing synergistic effects of these two GFs [147,149]. As for TGF- β 1, PDGF-BB combined to IGF-1 and delivered in liposomes showed promising results in the healing process of rat tooth sockets [150]. Spatiotemporal release of GFs from a delivery device can profoundly affect the efficacy of bone growth induction. Interestingly, microencapsulated IGF-1 with different release kinetics decreased the local inflammatory response and increased the expression of different GFs and new bone formation in sheep bone defect model [137].

7.9 Bone growth factors clinical applications

From bench to bedside, using cell/GFs functionalized materials to repair bone defects, after trauma or cancer resection, appears as a challenge in the reconstructive surgery domain. Such functionalized materials must fulfill multiple criteria including biocompatibility, biodegradability, osteoconduction, osteoinduction, as well as vascularization. Recombinant human (rh) BMP-2 and BMP-7 combined to a carrier (INFUSE, OP-1, Osigraft, InductOs) are the two commercially available BMPs for autograft replacement [34]. In July 2002, the FDA approved the first use of INFUSE Bone Graft Device [151] for spinal fusion procedures in skeletally mature patients with degenerative disc disease (www.accessdata.fda.gov). The latter, a combination of rhBMP-2 (1.5 mg/cm³) and an absorbable COL sponge (ACS) carrier, has since been linked to various health issues and adverse events [152,153]. In 2011, the same company received a “nonapprovable letter” from the FDA for AMPLIFY rhBMP-2 Matrix because of the high dose of rhBMP-2 (2.0 mg/mL) within the compression resistant carrier and the related adverse effects. As for rhBMP-2 and based on the encouraging clinical results [154], rhBMP-7 received, in October 2001, a limited FDA approval in the United States under a humanitarian device exemption to be used as “an alternative to autograft in recalcitrant long-bone nonunions where use of autograft is unfeasible and alternative treatments have failed.” It was introduced to the market as OP-1 in the United States and as Osigraft in Europe as a kit containing 3.5 mg of rhBMP-7, 1 g of type I bovine COL matrix, and 230 mg of the putty additive carboxymethylcellulose sodium (www.accessdata.fda.gov and www.ema.europa.eu). Based on controversy clinical results concerning the efficacy of commercialized rhBMP-7, the FDA rejected a premarket approval of OP-1 in April 2009. For marketing reasons, according to the marketing authorization holder, the sale of these products was withdrawn in the United States and Europe, in 2014 and 2015, respectively [155,156]. Optimistically, rhBMP-2 and rhBMP-7, are currently been tested in several clinical studies in combination with materials of interest summarized in a nonexhaustive list (Table 7.1, referred to www.clinicaltrials.gov).

When mixed with an osteoconductive scaffold such as β -TCP, rhPDGF-BB has been shown to improve clinical outcomes of many conditions including joint arthrodesis for foot/ankle problems [157] and periodontal bone defects [158–160]. Indeed, one of the largest clinical studies associating rhPDGF-BB/ β -TCP and foot/ankle joint arthrodesis was conducted in 37 clinical sites across the United States and Canada. The latter evidenced

TABLE 7.1 Nonexhaustive list of clinical studies associating bone morphogenetic protein (BMP) 2 and BMP-7 to materials of interest in bone tissue engineering.

Application	Phase	Device	Responsible party	Clinical trial identifier
Critical-sized bone defect	I	MSC/HaP-CaSO ₄ /rhBMP-2	Singapore University	NCT01725698
Decreased BMD	II	rhBMP-2/CPM	Pfizer	NCT00752557
Hip fractures				NCT00384358
Humerus closed fractures				NCT00384852
Symptomatic degenerative disc disease	NA	rhBMP-2 /ACS/Allograft Bone Dowel	Medtronic	NCT01494493
Degenerative disc disease		rhBMP-2 /ACS/INTER FIX™		NCT01491464 NCT01491451
		rhBMP-2 /BCP/TSRH spinal system		NCT01494454
		rhBMP-2/ACS/LT-CAGE		NCT01491373
Symptomatic degenerative disc disease	III	rhBMP-2/CRM/CD HORIZON		NCT00707265
Alveolar bone preservation	NA	rhBMP-2/ β -TCP	BioAlpha Inc.	NCT02714829
Adult femoral head necrosis	NA	rhBMP-7/autologous bone marrow	Lille University	NCT02655120
Tibial nonunion	IV	rhBMP-7 /demineralized bone matrix	Ghent University Hospital	NCT00551941

ACS, Absorbable collagen sponge; BCP, basic calcium phosphate; BMD, bone mineral density; CPM, calcium phosphate matrix; CRM, compression resistant matrix; HaP, hydroxyapatite; MSC, mesenchymal stem cells; NA, not applicable; rhBMP, recombinant human bone morphogenetic protein; TCP, tricalcium phosphate.

rhPDGF-BB/ β -TCP is at least as effective as autograft, as it offers the advantages of being safer and less painful for patients, especially when eliminating the bone graft harvest site (NCT00583375). The safety and efficacy of rhPDGF-BB/ β -TCP in the treatment of periodontal defects in short-term (up to 6 months, NCT00496847) and long-term (up to 36 months, NCT01530126) have been clinically demonstrated. Nevins and colleagues reported that rhPDGF-BB (0.3 mg/mL) in a synthetic scaffold matrix promotes long-term stable clinical and radiographic improvements as measured by composite outcomes for clinical attachment level gain and linear bone growth for patients possessing localized periodontal defects [160].

7.10 Conclusion and perspectives

The use of GFs has become the bull's eye of the bone tissue engineering field. Mitogenic, chemotactic, or osteoinductive, delivering GFs have been shown to be crucial

for mediating the interaction between cells/tissues and biomaterials in view of an effective regeneration of a functional bone tissue. In this chapter, we provided an overview of the “good face” but also the “ugly face” of GFs, in bone tissue engineering existing, in the literature. Indeed, the variation of parameters in studies (types of carrier, delivery approaches, GFs, cells, etc.) has led to controversy and sometimes to confusion but also, offered important information for future studies to generate a reliable list of requirements for a long-term bioactivity and delivery of BMP-2, which is considered to be the most potent osteogenic GF. Meanwhile and despite the optimism carried out from the studies mentioned above, most bone implant materials lack a well-established vasculature. The latter is crucial to maintain bone tissue in a “good shape” as it provides the local supply in oxygen and nutrients and increases the recruitment and survival of stem and osteoprogenitor cells. Mimicking bone autograft properties would require, in addition to the physicochemical features, a special mixture of GFs/cells crucial to recapitulate the bone regeneration process. VEGF is a master regulator of angiogenesis and is thought to have an important role in the effective coupling of angiogenesis and osteogenesis during both skeletal development and postnatal bone repair. Bone engineering approaches using VEGF were focused on its role in providing blood vessel network and the recruitment of progenitors. Indeed, delivering VEGF *in vivo* led to an increase in blood vessel density and an improvement in bone regeneration in many animal models [161,162]. In addition, VEGF is likely to act in synergy with osteoinductive GFs to improve osteogenesis [163]. However, VEGF doses should be seriously adapted and optimized, since non-physiological concentrations are thought to impair bone regeneration by hampering osteoblast differentiation and increasing bone resorption [163]. The latter would also depend on the: (1) type and structure of GFs carriers; (2) GFs ratio; (3) release manner and assessment time and not to forget; (4) the type of cells used in the study, since cells are known to exert proregenerative paracrine potential and constitute a vital source of VEGF.

Ultimately, the use of appropriate combinations of soluble/immobilized regulatory signals in a cell/material system is crucial to generate a well-structured, functional, and vascularized or even neurovascularized engineered bone tissue. Back to the salamander story, the remaining question would not be “how did he win the self-repair lottery and how did humans miss it?” but rather, how can we improve the role of each tissue engineering actor in order to reach the ideal regeneration condition?

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Conflict of interest

The authors declare no competing financial interest.

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Cell–biomaterial interactions: the role of ligand functionalization

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8.1 Introduction

The design of new biomaterial-based devices implies the optimization of their interactions with the living cells in the host tissue. Cells are in continuous communication with adjacent cells and microenvironment in order to maintain their homeostasis and function. Cells are characterized by a plethora of cell surface receptors (e.g., integrins) that can sense biochemical and biophysical signals outside the cell membrane. When stimulated, receptors activate intracellular signaling pathways that regulate gene expression and, as a result, cell behavior [1]. Essential signals come from their environment, both from neighboring cells and from the surrounding extracellular matrix (ECM) to which cells adhere. The ECM is a highly hydrated substrate composed by different types of molecules, such as collagens, elastic fibers, glycosaminoglycans (GAGs), and adhesive glycoproteins (e.g., laminin, fibronectin) [2]. These molecules are deposited by cells themselves, and different organs and tissues are characterized by different compositional and structural organization of their ECM, giving rise to their unique ECM arrangement [3]. Nowadays, ECM is known not to function just as passive support for cells, but it influences and controls cell behavior. In more detail, it supports the diffusion of soluble cytokines, mediates cell-cell interaction, and affects cell adhesion, proliferation, differentiation and possibly apoptosis, through its physical properties and chemical composition. Variation in ECM structure can have a great impact over cell functioning [2]. The first biomaterial-based devices exploited in regenerative medicine were based on inert materials, to avoid interaction with the host tissue and to reduce the probability of rejection. Currently, the trend is to develop biomaterials able to establish a specific communication with the resident cells of the implant area [4]. Such interactions are generally mediated by cell adhesive

molecules that can endorse cell attachment and activate specific cellular signaling pathways [1]. Furthermore, ligand functionalization is also exploited in nanomedicine for targeted drug release: the surface functionalization of drug-loaded nanoparticles (NPs) with selective ligands allows NP internalization by the target cells, minimizing off-target effects [5].

Considering these premises, the functionalization of biomaterials with specific ligands is of pivotal importance to impart the desired functionality to the final device. Ligands generally consist of brief peptides derived from ECM proteins. In the case of scaffolding biomaterials, the purpose is to mimic ECM stimuli for a fine control over cell adhesion, proliferation, and differentiation, enhancing tissue regeneration outcomes [6]. On the other hand, in nanomedicine, drug-loaded NPs may be decorated with surface ligands, that confer them the capability to interact with target cells, undergoing receptor mediated-endocytosis [7].

This chapter aims at presenting the most recent advances in biomaterial functionalization, with a focus on the surface functionalization of scaffolds and the bulk functionalization of hydrogels with cell adhesive specific peptides, as well as the functionalization of NPs to address cell targeting in drug release. Biomaterial development can assist the regeneration of human tissues/organs by a wide range of possible strategies; however, in this contribution our attention will be devoted to ligand functionalization of biomaterials for cardiac regeneration.

Cardiac failure is one of the primary causes of death and disability in the world [8]. A healthy heart comprises different cell types, including cardiomyocytes (CMs), cardiac fibroblasts, and endothelial cells (ECs). CMs retain the contractile activity of this organ but have an extremely low regeneration rate. Ischemic injury leads to the death of a huge number of CMs: functional tissue is replaced by cardiac scar, mainly populated by cardiac fibroblasts and composed of collagen, resulting in compromised heart function [9,10].

The currently available treatments to cardiac failure are invasive surgical interventions for implantation of ventricular assistance devices or heart transplantation, and/or pharmacological treatments unable to restore proper cardiac function.

Newly studied strategies to restore myocardial function are (1) the *in situ* grafting of CMs previously differentiated *in vitro* from patient-derived stem cells [11], (2) the stimulation of resident CMs to induce their proliferation [12,13], and (3) the generation of new CMs by the direct reprogramming of resident cardiac fibroblasts [14–16].

Tissue engineering (TE) exploits biomimetic materials to sustain cell function and cells/bioactive molecules delivery. Different supports have been developed, such as polymeric scaffolds recapitulating the mechanical, structural, and electrical properties of the native cardiac ECM [17], which can be functionalized to sustain cell growth and differentiation [18]. In addition, innovative injectable hydrogels can provide a three-dimensional (3D) environment to locally deliver cells, drugs, or biomolecules (e.g., growth factors) through a direct and non-invasive approach [17]. Finally, NPs can act as carriers to protect and deliver biomolecules to a precise site of action through specific ligand functionalization [5].

A synergic combination of biomaterials-mediated strategies with proper functionalization could improve treatments aimed at cardiac tissue regeneration.

8.2 Ligand functionalization in the design of bioactive hydrogels

In this section, general strategies for peptide functionalization of hydrogels are initially reported, followed by a description of biomimetic hydrogels for cardiac applications.

8.2.1 General functionalization strategies for hydrogels

The first application of hydrogels for biomedical purposes dates back to the 1960s, with the work published by Wichterle and Lím [19]. In its general definition, the term “hydrogel” identifies 3D polymeric networks with high swelling potential in a watery environment [20,21]. As a consequence of this high water content (hydrogels can reach swelling percentages in the order of hundreds %), these systems usually possess low stiffness and high deformability. These properties, which structurally mimic the natural ECM, make hydrogels highly promising systems for the design of scaffolds able to guide the regeneration of soft tissues. However, hydrogels often lack specific bioactive moieties and, thus, cannot exert precise control over cellular functions. For this reason, during the past few years, the design principles of hydrogels for TE have been mainly focused on making their forming materials biomimetic and bioactive. To this aim, ligand functionalization has been widely explored in the literature to graft polymers with bioactive/functional moieties, thus making them able to drive specific cell behaviors (i.e., adhesion, migration, proliferation, and differentiation).

Over the last few decades, an exhaustive investigation has been carried out on hydrogel functionalization with adhesive peptide sequences. In this regard, much research has been focused on the bulk grafting of peptides containing the adhesion motif arginine–glycine–aspartic acid (RGD) derived from fibronectin. For instance, Burdick and Anseth investigated the relationship between the RGD functionalization degree of poly(ethylene glycol) diacrylate (PEGDA) gels and the attachment of rat calvarial osteoblasts, proving that cell adhesion and spreading were significantly driven by RGD concentration [22]. Specifically, the number of attached cells significantly increased in RGD-modified gels compared to unmodified hydrogels at each analyzed time point, confirming RGD capability to enhance cell adhesion. Furthermore, a much higher cell density was observed upon the RGD concentration increase from 0.5 to 5 mM (Fig. 8.1), while cytoskeleton organization was detected only at 5 mM peptide concentration.

Another important aspect is the effect of a spacer sequence to peptide exposure and capability to exert its biological function. The first outcomes on this topic date back to 1998, when Hern and Hubbel compared unspaced and PEG₇₇ (3400 Da)-spaced RGD grafted to PEGDA backbone [23]. The presence of the spacer increased fibroblast spreading from 50% to 70% (only 5% spreading was observed in non-functionalized PEGDA gels), while PEGDA gels functionalized with unspaced RGD peptides did not exhibit any cell spreading when cells were cultured in serum-free conditions. Later, Wilson et al. further investigated the role of PEG spacer length on cell behavior, reporting that the concentration of RGD moieties required to support cell adhesion and spreading decreased with increasing PEG spacer length within the range PEG₅–PEG₇₇, thus suggesting that longer spacers make bioactive ligands more available for interactions with cells [24].

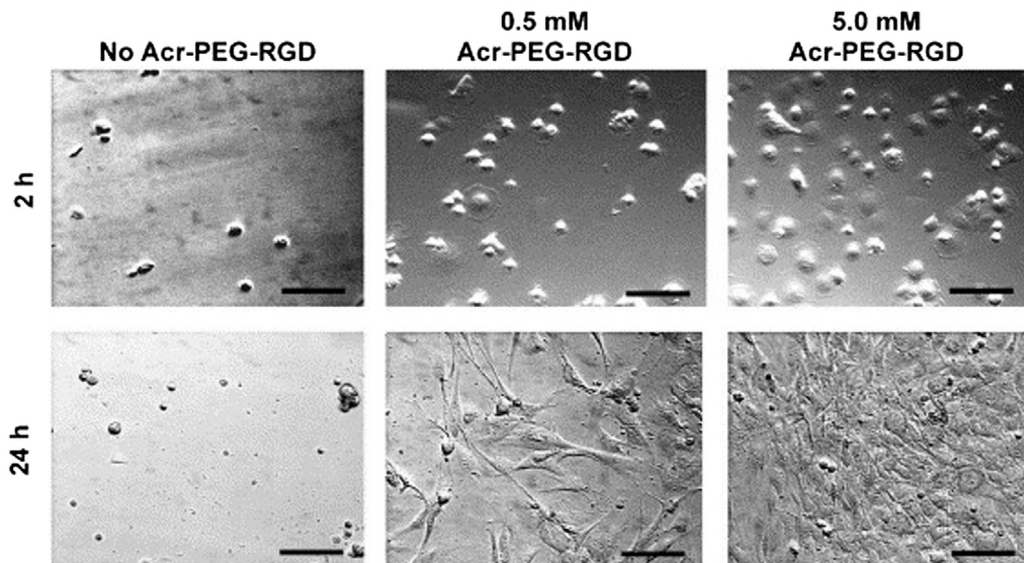


FIGURE 8.1 Light micrographs of attached osteoblasts after 2 and 24 h culture on PEGDA gels with no adhesive peptides (left images), with 0.5 mM RGD (central images), and with 5 mM RGD (right images). PEGDA, Poly (ethylene glycol)diacrylate; RGD, arginine–glycine–aspartic acid. Source: Reprinted with permission from Burdick JA, Anseth KS. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials* 2002;23:4315–23. Available from: [https://doi.org/10.1016/S0142-9612\(02\)00176-X](https://doi.org/10.1016/S0142-9612(02)00176-X). ©2002, from Elsevier.

In addition to the spatial control on RGD sequence exposure, its temporally controlled presentation has been shown to influence cell behavior, allowing cells to exert specific functions. For instance, Lee et al. developed PEGDA hydrogels functionalized with RGD sequences modified with a light-sensitive moiety on the carboxylic terminal group of aspartic acid [25]. This caging group was successfully released upon hydrogel exposure to UV light (wavelength within 350–365 nm), thus making the RGD sequences available for interaction with the surrounding cells (Fig. 8.2).

The potential of the approach was demonstrated by implanting the hydrogels subcutaneously in mice: successful exposure of RGD sequences was achieved through non-invasive transdermal UV-light irradiation for 10 minutes, with no skin damage. Moreover, timely triggered exposure of RGD moieties turned out to significantly impact the modulation of chronic inflammatory response and fibrosis: RGD exposure at 7 or 14 days from implantation induced the formation of a fibrotic capsule with approx. 50% lower thickness than hydrogels subjected to UV irradiation and RGD exposure immediately after implantation. RGD exposure could also be regulated by using enzyme-cleavable peptide sequences. In this regard, Salinas and Anseth demonstrated the importance to trigger RGD sequence exposure in guiding human mesenchymal stem cell (hMSC) chondrogenesis, in a similar way as in natural stem cell niches [26,27]. hMSCs cultured in RGD-cleavable hydrogels produced 10-fold and 4-fold higher amounts of GAGs and collagen type II, respectively, compared to hydrogels with uncleavable RGD moieties [27].

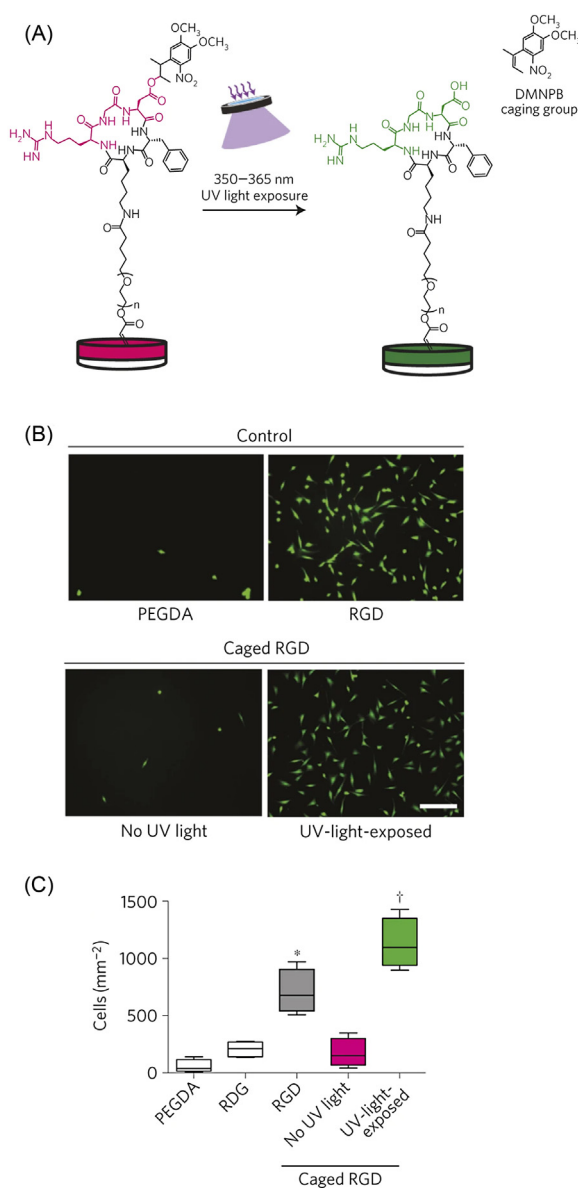


FIGURE 8.2 (A) Preparation of RGD-functionalized PEGDA hydrogel: removal of the photolabile protecting group 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl ester through system exposure to UV light. (B) Fluorescently labeled cells cultured on virgin PEGDA (control), free RGD-functionalized PEGDA, and caged RGD-functionalized PEGDA exposed or not-exposed to UV light. (C) Adherent cell density on virgin PEGDA, free RGD-functionalized PEGDA, free RGD-functionalized PEGDA (scrambled peptide), and caged RGD-functionalized PEGDA exposed or not-exposed to UV light. *PEGDA*, Poly(ethylene glycol)diacrylate; *RGD*, arginine–glycine–aspartic acid; *RDG*, arginine–aspartic acid–glycine. Source: Reprinted with permission from Lee TT, García JR, Paez JI, Singh A, Phelps EA, Weis S, et al. Light-triggered in vivo activation of adhesive peptides regulates cell adhesion, inflammation and vascularization of biomaterials. *Nat Mater* 2015;14:352–60. Available from: <https://doi.org/10.1038/nmat4157>. ©2015, Springer Nature Publishing AG.

To further increase hydrogel adhesiveness and to better mimic ECM composition, Gould et al. designed a new thiol-ene hydrogel exposing a combination of peptide sequences [28]. Specifically, with the final aim to assess the role exerted by biochemical cues in the formation of myofibroblasts from valvular interstitial cells (VICs), the authors developed a PEG-based hydrogel containing different amounts of P15,

VGAPG, and RGDS (glycine-threonine-proline-glycine-proline-glutamine-glycine-isoleucine-alanine-glycine-glutamine-arginine-glycine-valine-valine, valine-glycine-valine-alanine-proline-glycine and arginine-glycine-aspartic acid-serine, respectively) derived from collagen type I, elastin and fibronectin, respectively. The exposure of RGDS alone allowed a moderate α -smooth muscle expression (α -SMA), while the combination with elastin- and collagen-derived sequences significantly increased α -SMA expression, if compared to that obtained considering the whole ECM (control condition). These findings suggested that only those peptides belonging to the ECM were effectively responsible for VIC activation into myofibroblasts. Depending on the target cell behavior, other ligands have been also tested, such as the laminin-derived RKRLQVQLSIRT syndecan-1 binding ligand (arginine-lysine-arginine-leucine-glutamine-valine-glutamine-leucine-serine-isoleucine-arginine-threonine) to regulate the hemostatic functions of valve ECs [29] and the fusion proteins EphA5-Fc and EphrinA5-Fc which are involved in the regulation of both insulin secretion and β cell communication pathways [30]. With the aim of triggering cell infiltration and migration in the damaged area, matrix metalloproteinases (MMPs) activity has been usually mimicked. In detail, such enzymes are the main players in ECM degradation, allowing cells to migrate toward specific sites to organize a new tissue. A wide variety of MMP-sensitive substrates has been isolated from animal and human proteins and the possibility to finely modulate their degradation kinetics by changing their amino acid composition has been already reported by Nagase and Fields in 1996 [31]. Adhesivity and enzyme-sensitivity have thus been combined in a sole hydrogel to ensure MMP- and integrin-mediated cell homing and migration within gel network. For instance, Lutolf et al. demonstrated that human fibroblasts migrate within PEG hydrogels at a rate depending on MMP-mediated degradation, concentration of attachment ligands, and degree of crosslinking [32]. Similarly, Mann et al. designed a PEG hydrogel exposing both adhesive and proteolytically degradable peptides in order to guide hydrogel degradation by tissue formation processes [33]. Specifically, the authors selected the previously mentioned RGD sequence to improve cell adhesion and LGPA (leucine-glycine-proline-alanine) and 9-mer of alanine enzyme-sensitive peptides to achieve degradation by collagenase and elastase, respectively. Results showed that in the absence of one of these sequences, cell migration through the gel was not observed. Cell binding to adhesive peptides is necessary for cell migration and secretion of proteolytic enzymes; furthermore, the formation of pores facilitates cell migration mechanism. The same approach was exploited later by Phelps et al. that designed bioactive PEG hydrogels decorated with pendant adhesive RGD peptides and cross-linked with protease-sensitive GCRDVPMSMRGGDRCG peptide (glycine-cysteine-arginine-aspartic acid-valine-proline-methionine-serine-methionine-arginine-glycine-glycine-aspartic acid-arginine-cysteine-glycine) [34]. Kyburz and Anseth exploited a light-initiated thiol-ene reaction to design hMSC-embedded PEG-based gels of varying susceptibility to MMPs (by tuning the crosslinking degree) and adhesion properties (by modulating the amount of grafted CRGDS (cysteine-arginine-glycine-aspartic acid-serine) peptide sequences) [35]. Thiol groups of cysteine residues were exploited to react with PEG macromolecules functionalized with norbornene moieties. In order to make the gels degradable in response to biochemical stimuli, peptide sequences susceptible to MMPs 1, 2, 3, 8, and 9 which are secreted by hMSCs were used to crosslink the systems.

Another important aspect is the design of immunomodulatory hydrogels to avoid the infiltration of proinflammatory cytokines and foreign body reaction. To this aim,

antiinflammatory peptides have been used. For instance, Su et al. developed a PEG-based hydrogel for pancreatic islet encapsulation acting as a barrier against the infiltration of immunocytes and low molecular weight inflammatory factors due to the exposure of an inhibitory peptide for islet cell surface interleukin-1 (IL-1) receptor (IL-1RIP, phenylalanine-glutamic acid-tryptophan-threonine-proline-glycine-tryptophan-tyrosine-glutamine-proline-tyrosine-NH₂, FEWTPGWYQPY-NH₂) [36]. IL-1RIP functionalized PEG hydrogels reduced the death of loaded cells to 60% compared to not-modified gels, confirming peptide function against inflammation. Moreover, the coexposure of IL-1RIP and RGD sequence further enhanced anticytokine effects (Fig. 8.3). On the other hand, grafted peptides did not reduce cell ability to secrete insulin in response to changes in glucose concentration; the presence of IL-1RIP sequence further induced insulin secretion by encapsulated cells.

During the same years, Lin et al. carried out similar investigations designing a PEG-based hydrogel functionalized with the highly specific tumor necrosis factor α (TNF- α) binding sequence, WP9QY (YCWSQYLCY, tyrosine-cysteine-tryptophan-serine-glutamine-tyrosine-leucine-cysteine-tyrosine) [37]. The ability of WP9QY peptide to preserve the viability of loaded cells was demonstrated using three different cell types, that is, adrenal pheochromocytoma cells from rats (PC12s), mouse pancreatic islets, and hMSCs. Results revealed that peptide-functionalized PEG gels prolonged PC12 cell and mouse islet survival and functionality, thus demonstrating the capability of the designed systems to modulate local inflammation. For what concerns hMSC encapsulation, WP9QY peptide grafting hindered hMSC proliferation induced by TNF- α and did not alter their potential to undergo osteogenic differentiation.

8.2.2 Peptide functionalization of hydrogels for cardiac tissue engineering

Hydrogels have been widely investigated in cardiac regeneration over the last few decades. According to the classification proposed by Reis et al. [38], injectable hydrogels are

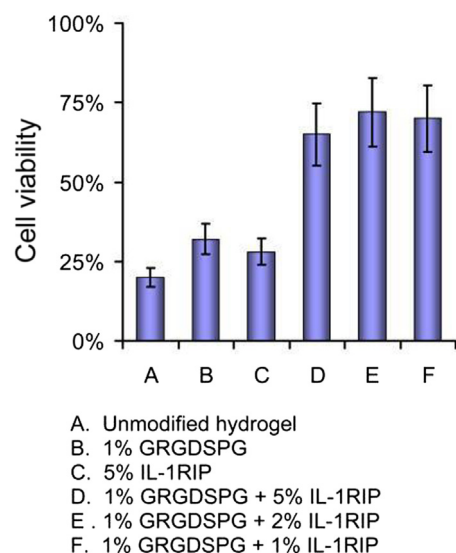


FIGURE 8.3 Pancreatic cells encapsulated in ligand-functionalized hydrogels to investigate anti-inflammatory peptides preservation against cell death induced by cytokines. 24 h post encapsulation, cells were treated with IL-1 β , TNF- α , and INF- γ for 2 h and cell death was investigated through LIVE/DEAD assay. Cell encapsulation within RGD- and IL-1RIP-modified hydrogels significantly increased their viability compared to cells encapsulated in hydrogels exposing only one or no peptide. RGD, Arginine-glycine-aspartic acid; TNF- α , tumor necrosis factor α ; INF- γ , interferon gamma. Source: Reprinted with permission from Su J, Hu B-H, Lowe WL, Kaufman DB, Messersmith PB. Anti-inflammatory peptide-functionalized hydrogels for insulin-secreting cell encapsulation. *Biomaterials* 2010;31:308–14. Available from: <https://doi.org/10.1016/j.biomaterials.2009.09.045>. ©2010, Elsevier.

usually applied in cardiac TE to (1) induce endogenous repair through the recruitment of endogenous cells, the retention of cell survival and the stimulation of cell proliferation, differentiation and neovascularization processes; (2) promote exogenous regeneration through cell therapy approaches (the hydrogel is used as cell carrier); or (3) provide a physico-mechanical support to the injured heart region with the aim to keep and restore wall thickness and heart geometry and, as a consequence, improve heart function (acellular hydrogels). Beyond this classification, hydrogels are often designed to be multifunctional to make them able to exert more than one function.

Hydrogels aiming at inducing and guiding endogenous or exogenous cardiac regeneration are usually based on bioactive biomaterials provided with specific moieties along their backbone or able to release biomolecules (e.g., angiogenic, antiapoptotic, immunomodulatory molecules [39]) to the injured area. Bioactive biomaterials are usually obtained by grafting properly selected peptide sequences to native polymer backbone through functionalization procedures. The variety of peptides investigated to functionalize hydrogels for cardiac application can be categorized into three main classes: (1) antiapoptotic and cardioprotective sequences (e.g., QHREDGS (glutamine-histidine-arginine-glutamic acid-aspartic acid-glycine-serine), glutathione); (2) adhesive and proangiogenic peptides (e.g., RGD, GFOGER (glycine-phenylalanine-hydroxyproline-glutamic acid-arginine), YPHIDSLGHWRR (tyrosine-proline-histidine-isoleucine-aspartic acid-serine-leucine-glycine-histidine-tryptophan-arginine-arginine, RoY) peptide); and (3) cardiac phenotype inducers (e.g., Notch1 ligand Jagged1 mimicking peptide).

In cardiac TE, cellular therapies exploiting cardiac progenitor cells (CPCs) represent a promising strategy to induce infarcted cardiac tissue restoration due to their ability to differentiate toward the cardiac, endothelial, and vascular smooth muscular phenotypes as well as their paracrine effects [40]. However, poor retention of injected cells and the low survival in the hostile infarcted environment have limited the use of CPCs in the clinics. Bioactive hydrogels could overcome these drawbacks, providing the cells with a friendly biomimetic environment. In this context, self-assembling peptide hydrogels were functionalized with a peptide mimicking the Notch1 ligand Jagged1 (RJ, H₂N-CDDYYYGFGCNK FCRPR-OH, H₂N-cysteine-aspartic acid-aspartic acid-tyrosine-tyrosine-tyrosine-glycine-phenylalanine-glycine-cysteine-asparagine-lysine-phenylalanine-cysteine-arginine-proline-arginine-OH) to trigger the Notch signaling pathway, which is activated in the early stages of cardiac development as well as in CPC survival and differentiation pathways [41,42]. Injection of CPC-loaded or -free RJ-functionalized hydrogels in murine model of myocardial infarction improved heart functionality recovery (improvements in cardiac output, ejection fraction, stroke volume, stroke work), contractility, and neovascularization of the infarcted area, accompanied with decreased fibrosis and increased CM cell cycle activity (increased expression of Ki67) compared to untreated animals or rats subjected to injection of virgin hydrogels or hydrogels grafted with a nonfunctional peptide sequence (RS, H₂N-RCGPDCFDNYGRYKYCF-OH, H₂N-arginine-cysteine-glycine-proline-aspartic acid-cysteine-phenylalanine-aspartic acid-asparagine-tyrosine-glycine-arginine-tyrosine-lysine-tyrosine-cysteine-phenylalanine-OH) (Fig. 8.4).

With the aim to enhance CPC retention within the hydrogels, thus protecting them from the hostile environment of the infarcted area, Bhutani et al. [43] encapsulated CPCs into PEG-based hydrogels grafted with the RGD and the GFOGER (collagen-mimetic

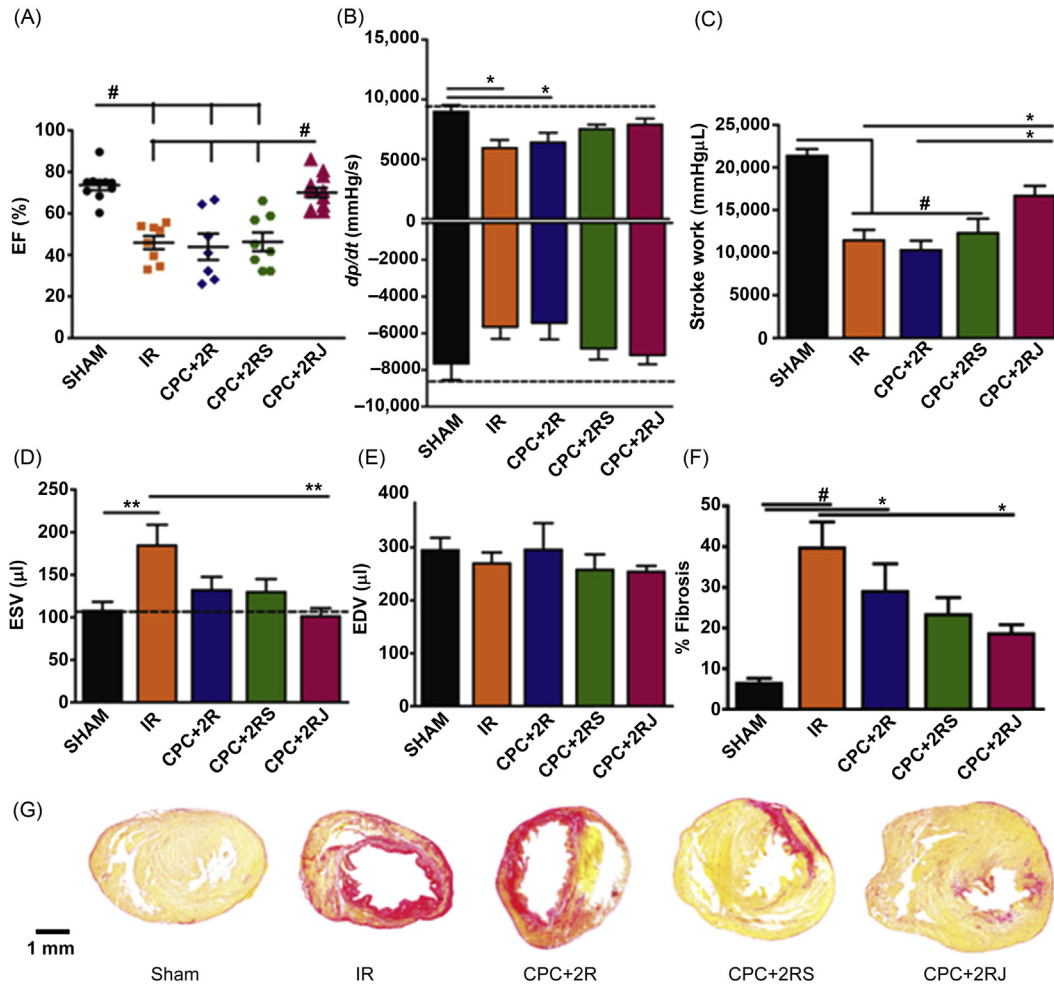


FIGURE 8.4 Injection of CPC-loaded SAP hydrogel (2% w/v) functionalized or not-functionalized with peptides (2R: not-functionalized, 2RJ: functionalized with RJ, 2RS: functionalized with RS). Comparison with sham-operated animals (SHAM, i.e., subjected to placebo surgery) or animals subjected to ischemia-reperfusion procedure (IR). (A) EF%, (B) rate of left ventricular pressure increase (dp/dt), (C) stroke work, (D) ESV, (E) EDV, (F) percentage of fibrosis (% Fibrosis), (G) Pico-Sirius red-stained heart sections. CPC, Cardiac progenitor cell; EDV, end diastolic volume; EF, ejection fraction; ESV, end systolic volume; SAP, self-assembling peptide. Source: Reprinted with permission from Boopathy AV, Che PL, Somasuntharam I, Fiore VF, Cabigas EB, Ban K, et al. The modulation of cardiac progenitor cell function by hydrogel-dependent Notch1 activation. *Biomaterials* 2014;35:8103–12. Available from: <https://doi.org/10.1016/j.biomaterials.2014.05.082>. ©2014, Elsevier.

peptide) sequences. Both RGD- and GFOGER-grafted gels exhibited higher cell adhesion compared to hydrogels grafted with the nonadhesive RDG sequence used as control (approx. five-fold increase in cell adhesion). Surprisingly, *in vitro* CPCs underwent cardiac differentiation accompanied by a reduction in the secretion of reparative paracrine factors

when cultured in GFOGER-grafted hydrogels, probably as a consequence of their biomimetic mechanical properties and the biological signaling pathways initiated by GFOGER sequence. However, unexpectedly, *in vivo* the best recovery in cardiac function was observed upon injection of RDG-exposing hydrogels loaded with CPCs, which also showed the best retention of transplanted cells. These findings have thus opened a new chapter in the field, suggesting that (1) cellularized hydrogels exposing adhesive peptides along their backbone could elicit a higher immune response upon injection *in vivo* leading to hydrogel degradation and possible cell death, and (2) grafted adhesive peptides could block CPC integrins thus limiting their engagement with the surrounding tissue and engraftment. Although the role of adhesion ligands on hydrogels for cell delivery to the infarcted area has been criticized, their use in acellular hydrogels has been reported to favor ventricular function recovery and angiogenesis (significantly higher arteriole density compared to unmodified gel) as a consequence of integrin-ligand interactions that stimulate tissue regeneration [44].

The capability of RGD-functionalized hydrogels to interact with cell receptors and enhance cell adhesion has been also exploited by Plouffe et al. to design a new RGD-functionalized alginic acid coating able to control the capture and release of cardiac fibroblasts within a microfluidic system [45]. RGD exposure improved the capture of cardiac fibroblasts flowing within the system (two-fold higher compared to unmodified alginic acid hydrogel), meanwhile hydrogel dissolution under mild conditions made cell release easy, thus providing viable cells for further applications.

The goal of improving angiogenesis and cardiac repair upon myocardial infarction was also achieved through functionalization of hydrogel forming material with the so-called RoY peptide [46]. RoY peptide has been reported to interact with GRP78 receptor which is overexpressed by vascular ECs in hypoxia conditions (a typical condition of the infarcted heart), thus activating cell survival and proliferation pathways. As a matter of fact, RoY-functionalized chitosan (CH) chloride-based gels promoted the survival and proliferation of human umbilical vein ECs as well as their organization into tubular constructs. Results obtained *in vitro* were further confirmed *in vivo* upon gel injection in a myocardial infarction rat model: animals treated with RoY-grafted hydrogels showed increased angiogenesis and, as a consequence, improved recovery of cardiac function compared to rats subjected to injection of saline solution or virgin gel (Fig. 8.5).

The same bulk material was also used to design antioxidant hydrogels with scavenging activity toward the reactive oxygen species overproduced after a myocardial infarction, which represent one of the main obstacles for a successful cardiac regeneration [47]. In detail, scavenging potential was provided to CH chloride by grafting glutathione (glutamic acid-cysteine-glycine tripeptide), which has been reported to favor cell adhesion and protect cells from oxidative stress [48,49].

Cardioprotective and prosurvival features were also obtained by grafting CH with the integrin-binding motif of angiopoietin-1 growth factor (QHREDGS) that was found to promote CM adhesion and survival in a similar way as the full-length molecule. QHREDGS-grafted CH hydrogels promoted CM survival and adhesion at similar levels as RGDS-grafted hydrogels (used as positive control). However, these newly designed hydrogels showed a superior ability of preserving cells from apoptosis,

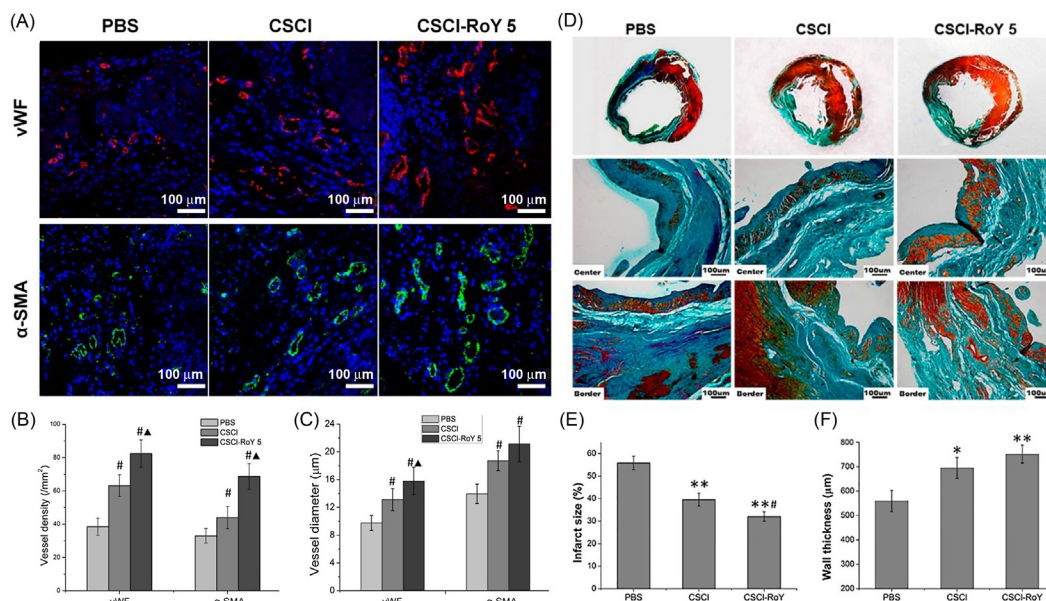


FIGURE 8.5 Angiogenesis in the infarcted area of rats 28 days postsurgery: (A) myocardial sections from hearts of animals treated with saline (PBS, phosphate buffered saline, pH 7.4), virgin CSCI, and CSCI-RoY 5 stained with vascular specific antibodies [vWF (red) and α-SMA (green)]; (B and C) vessel density and diameter. Myocardial structures of the infarcted region 28 days postsurgery: (D) cardiac tissue stained with Masson's trichrome staining; (E and F) quantitative evaluation of infarct size and infarct wall thickness. CSCI, Chitosan chloride gel; CSCI-RoY 5, RoY-functionalized chitosan chloride gel; vWF, Von Willebrand factor; α-SMA, α-smooth muscle expression. Source: Reprinted with permission from Shu Y, Hao T, Yao F, Qian Y, Wang Y, Yang B, et al. RoY peptide-modified chitosan-based hydrogel to improve angiogenesis and cardiac repair under hypoxia. *ACS Appl Mater Interfaces* 2015;7:6505–17. Available from: <https://doi.org/10.1021/acsami.5b01234>. ©2015, American Chemical Society.

promoting both elongation and contractile apparatus assembly [50,51]. *In vivo*, a peptide concentration-dependent response was observed: a higher number of recruited myofibroblasts and viable CMs were detected after subcutaneous injections of CM-loaded hydrogels prepared from CH grafted with a higher amount of QHREDGS (approx. 650 nmol of peptide per gel mL) [52]. In addition, a higher number of beating CMs were obtained with increasing peptide grafting to CH chains. Hydrogels injected in a myocardial infarction rat model remained *in situ* for approximately 3 weeks and induced significant improvements in cardiac morphological and functional features compared to control conditions (i.e., animal injected with PBS) and not-functionalized hydrogels. In more detail, scar thickness, fractional shortening, and ejection fraction improved by 53%, 35%, and 62%, respectively, meanwhile a 34% decrease in fractional scar area was observed [53].

All the above studies demonstrated the key role of ECM-like hydrogels in properly guiding the fate of encapsulated and host cells. To this purpose, one widely adopted strategy is to graft bioactive ligands to natural and synthetic hydrogels, exploiting their exposed functional groups, as summarized in Table 8.1.

TABLE 8.1 Widely adopted peptide ligands to mimic the natural extracellular matrix in the design of functional hydrogels.

Peptide sequence	Biological function	Hydrogel material	application
<i>General applications</i>			
RGD	Adhesiveness	PEG	Hydrogel loaded with osteoblasts [22]
RGD	Adhesiveness	PEGDA	Evaluation of spacer length on peptide efficacy [23]
RGD	Adhesiveness	PEGDA	Hydrogels with biological function controlled through UV irradiation [25]
RGD + MMP-13 linker	Adhesiveness and enzymatic sensitivity	PEGDA	Hydrogel sensitive to enzymatic degradation for hMSC differentiation in chondrocytes [27]
GRGDSPC + GCRDVPMSMRGGDRCG	Adhesiveness and enzymatic sensitivity	PEG	Hydrogel sensitive to protease enzymatic activity [34]
RGDS + VGVAPG + P15	Adhesiveness	PEG	Hydrogels exposing multiple peptide sequences for VIC activation into myofibroblasts [28]
RKR	Hemostatic control	PEGDA	Controlled hemostatic function of valve endothelial cells [29]
EphA5-Fc	Control over insulin secretion	PEG	Controlled insulin secretion from encapsulated β cells [30]
EphrinA5-Fc			
RGD + LGPA	Adhesiveness and enzymatic sensitivity	PEG	Hydrogels sensitive to collagenase [33]
RGD + 9AK	Adhesiveness and enzymatic sensitivity	PEG	Hydrogels sensitive to elastase [33]
RGD + KCGPQG↓IWGQCK	Adhesiveness and enzymatic sensitivity	PEG-norbornene	hMSC-loaded hydrogels with increased adhesiveness and controlled degradation [35]
RGD + IL-1RIP	Adhesiveness and antiinflammatory properties	PEG	Antiinflammatory hydrogels for pancreatic islet transplantation [36]
WP9QY	Antiinflammatory properties	PEG	Cell-loaded hydrogels with enhanced protection against TNF- α -induced damage [37]
<i>Cardiac applications</i>			
RGD	Adhesiveness	Alginate acid	Adhesive coating for microfluidic systems [45]
Jagged 1-mimetic peptide	Trigger for Notch	Self-assembling peptides	Self-assembling peptide hydrogels to activate Notch signaling pathway [42]

(Continued)

TABLE 8.1 (Continued)

Peptide sequence	Biological function	Hydrogel material	application
RoY peptide	Angiogenesis	Chitosan chloride	Injectable bioactive hydrogels to stimulate angiogenesis and improve cardiac function after myocardial infarction [46]
Angiopoietin-1 peptide QHREDGS	Cardioprotection and prosurvival	Photocrosslinkable azidobenzoic acid modified chitosan	Hydrogels for heart cells attachment and survival [51]
		Chitosan/collagen blend	Injectable hydrogels for cardiac cell culture and delivery [52] Acellular hydrogel for cardiac functional and morphological recovery upon myocardial infarction [53]
RGD	Angiogenesis	Alginate	Cell-free injectable gels with proangiogenic properties [44]
GFOGER	Adhesiveness	PEG	CPC-loaded hydrogels to be locally injected in the infarcted region [43]
RGD	Adhesiveness	PEG	CPC-loaded hydrogels to be locally injected in the infarcted region [43]
Glutathione	Reactive oxygen species scavenging	Chitosan	Injectable hydrogel to suppress oxidative stress damages [47]
GRGDSPC + GCRDVPMSMRGGDRCG	Adhesiveness, enzymatic sensitivity, triggered growth factor release	PEG	Hydrogels sensitive to protease cleavage for localized delivery of growth factors in infarcted area [34]

CPC, Cardiac progenitor cell; hMSC, human mesenchymal stem cell; MMP, matrix metalloproteinase; PEG, poly(ethylene glycol); PEGDA, poly(ethylene glycol)diacrylate; RGD, arginine–glycine–aspartic acid; TNF- α , tumor necrosis factor α ; VIC, valvular interstitial cell; GRGDSPC, glycine-arginine-glycine-aspartic acid-serine-proline-cysteine; GCRDVPMSMRGGDRCG, glycine-cysteine-arginine-aspartic acid-valine-proline-methionine-serine-methionine-arginine-glycine-glycine-aspartic acid-arginine-cysteine-glycine; RKR, arginine-lysine-arginine; KCGPQG↓IWGQCK, lysine-cysteine-glycine-proline-glutamine-glycine↓isoleucine-tryptophan-glycine-glutamine-cysteine-lysine; P15, glycine-threonine-proline-glycine-proline-glutamine-glycine-isoleucine-alanine-glycine-glutamine-arginine-glycine-valine-valine; VGVAPG, valine-glycine-valine-alanine-proline-glycine; RGDS, arginine-glycine-aspartic acid-serine; RKRLQVQLSIRT, arginine-lysine-arginine-leucine-glutamine-valine-glutamine-leucine-serine-isoleucine-arginine-threonine; LGPA, leucine-glycine-proline-alanine; WP9QY, tyrosine-cysteine-tryptophan-serine-glutamine-tyrosine-leucine-cysteine-tyrosine; IL-1RIP, phenylalanine-glutamic acid-tryptophan-threonine-proline-glycine-tryptophan-tyrosine-glutamine-proline-tyrosine-NH₂; Jagget 1-mimetic peptide, H₂N-cysteine-aspartic acid-aspartic acid-tyrosine-tyrosine-tyrosine-glycine-phenylalanine-glycine-cysteine-asparagine-lysine-phenylalanine-cysteine-arginine-proline-arginine-OH; Roy, tyrosine-proline-histidine-isoleucine-aspartic acid-serine-leucine-glycine-histidine-tryptophan-arginine-arginine; QHREDGS, glutamine-histidine-arginine-glutamic acid-aspartic acid-glycine-serine; GFOGER, glycine-phenylalanine-hydroxyproline-glutamic acid-arginine; glutathione, glutamic acid-cysteine-glycine; 9AK, alanine-alanine-alanine-alanine-alanine-alanine-alanine-alanine-alanine-lysine.

Finally, in a different approach, ligand-receptor interactions have been also exploited to drive the sol-to-gel transition [54] and peptide sequences have been used as crosslinking moieties among polymer chains [34,35,55]. This latter approach has been exploited to design hydrogels susceptible to a triggered degradation by specific enzymes with the final aim of locally releasing cells or biomolecules. For instance, Salimath et al. loaded

hepatocyte and vascular endothelial growth factors (HGF and VEGF, respectively) into PEG hydrogels grafted with RGD moieties and crosslinked with a protease-cleavable peptide sequence (GCRDVPMSMRGGDRCG, glycine-cysteine-arginine-aspartic acid-valine-proline-methionine-serine-methionine-arginine-glycine-glycine-aspartic acid-arginine-cysteine-glycine) and delivered them to the border zone of the infarcted region in rat myocardium [55]. HGF and VEGF release from the hydrogel increased angiogenesis and stem cell recruitment from the surrounding tissue, and significantly decreased fibrosis compared to the injection of a single growth factor (either encapsulated in the hydrogel or delivered as free growth factor solution), virgin PEG hydrogel, or HGF/VEGF mixture (Fig. 8.6).

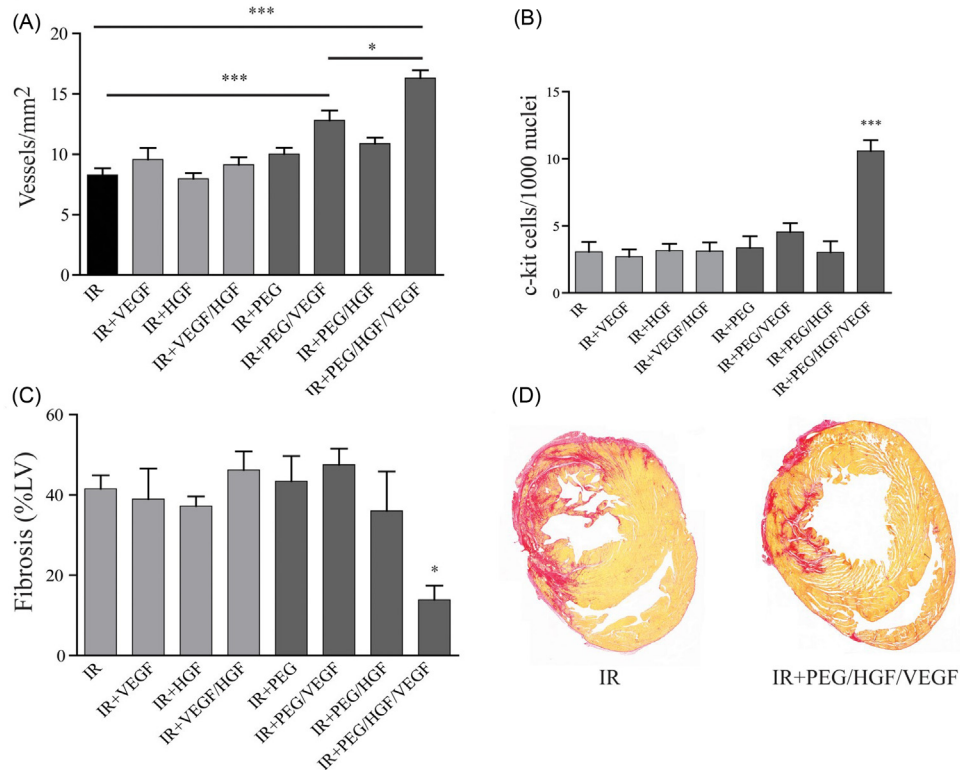


FIGURE 8.6 Evaluation of angiogenesis (expressed vessels/mm²) (A), c-kit positive cells (i.e., progenitor cells) (B), and fibrosis (C) expressed as percentage of the total LV area in the infarcted region of rats, assessed 21 days post myocardial infarction. (D) Representative heart sections stained for collagen (index of fibrosis) with picrosirius red (red stain, 206×). Group nomenclature: IR: rats subjected to IR surgery (control); IR + VEGF: IR-rats treated with VEGF aqueous solution; IR + HGF: IR-rats treated with HGF aqueous solution; IR + VEGF/HGF: IR-rats treated with VEGF/HGF aqueous solution; IR + PEG: IR-rats treated with PEG gel; IR + PEG/VEGF: IR-rats treated with VEGF-loaded PEG gel; IR + PEG/HGF: IR-rats treated HGF-loaded PEG gel; IR + PEG/VEGF/HGF: IR-rats treated with VEGF/HGF-loaded PEG gel. HGF, Hepatocyte growth factor; IR, ischemia-reperfusion; LV, left ventricular; PEG, poly(ethylene glycol); VEGF, vascular endothelial growth factor. Source: Adapted from Salimath AS, Phelps EA, Boopathy AV, Che P, Brown M, García AJ, et al. Dual delivery of hepatocyte and vascular endothelial growth factors via a protease-degradable hydrogel improves cardiac function in rats. *PLoS One* 2012;7:e50980. Available from: <https://doi.org/10.1371/journal.pone.0050980>.

8.3 Ligand surface functionalization in the design of scaffolds and implants

Scaffolds play a crucial role in TE and act as a support for the growth of new tissues, promoting cell adhesion and proliferation. For this reason, TE is aimed at designing and fabricating scaffolds mimicking the ECM of the tissue to be engineered. Biomimetic mechanical properties, chemical composition, and architecture are the target requirements for TE scaffolds [56]. Considering the ECM composition, different natural polymers, such as proteins and polysaccharides, have been used as scaffold materials; however, they suffer from poor mechanical properties and stability in physiological environment. On the other hand, the main advantages of synthetic polymers are their superior mechanical properties, tailored degradation rate, and processability; however, they do not possess any functionality recognized by the cells for integrin activation [57]. “Bioartificial materials” are materials based on synthetic and natural polymers or bioactive peptides combining their properties. Surface modification approaches are among the possible methods to introduce bioactive molecules on the surface of synthetic polymer substrates, without affecting the material bulk properties [58].

As discussed in the previous sections, RGD sequence has been widely investigated with the aim to enhance cell adhesion in many biomedical fields, such as bone and cardiovascular applications [59]. Titanium (Ti) and its alloys are important materials in orthopedic implant surgery, due to their biocompatibility with tissues and excellent mechanical properties; however, in clinical practices the osteointegration of orthopedic implants is often incomplete, resulting in a high risk of implant loosening over time. RGD peptide may improve the implant osteointegration [60]. Chua et al. [61] designed a multilayered coating able to combine antibacterial properties and cell adhesion. They performed a layer-by-layer coating on Ti substrate surface using hyaluronic acid (HA) and CH as polyelectrolytes. After the deposition of five HA/CH bilayers, with CH as the outermost layer, they covalently grafted RGD peptide via carbodiimide chemistry. Results demonstrated that the HA/CH coating showed antibacterial efficacy and, only in the case of RGD covalent grafting, the surface modification showed a positive influence on osteoblast adhesion and proliferation.

This section will be focused on the surface functionalization of cardiovascular implants, including coronary stents and scaffolds for myocardial regenerations.

Surface-induced thrombosis and in-stent restenosis cause the major clinical failures of cardiovascular stents. The formation of a functionally intact endothelium on the implant could inhibit growth of neointimal tissue after percutaneous coronary intervention and prevent thrombosis [62]. Many efforts were addressed to enhance and accelerate stent re-endothelialization, by surface functionalization with specific peptides. Li et al. [63] synthesized a Gly–Arg–Gly–Asp–Ser–Pro (GRGDSP) peptide coupled with photoactive 4-benzoylbenzoic acid, that was grafted on the surfaces of poly(carbonate urethane)s (PCUs) by UV irradiation, with the purpose to improve re-endothelialization of small-diameter vascular grafts. The proliferation and spreading of adherent ECs on modified PCU surfaces increased with increasing the concentration of the peptide. Moreover, the retention of ECs on the functionalized PCU was higher compared to the uncoated PCU under flow shear stress conditions. In conclusion, GRGDSP grafted on the surface of small-diameter vascular grafts and functional tissue engineered small-diameter blood vessels was

demonstrated to be effective in enhancing re-endothelialization. However, RGD-peptide is recognized by approximately half of the integrin cell receptors and it has been found to promote platelets, ECs, and smooth muscle cells (SMCs) adhesion. Therefore RGD-based peptides are not able to support *in vivo* selective adhesion and proliferation of ECs [64]. The REDV (arginine–glutamic acid–aspartic acid–valine) fibronectin-derived peptide is recognized by $\alpha 4\beta 1$ integrins and has been reported to selectively promote EC adhesion and spreading over SMCs and platelets [65]. Ceylan et al. [66] developed a peptide-based self-assembled nanofibrous coating functionalized with REDV. Their results showed that REDV functionalization provided selective growth of ECs on the stainless steel surface, as shown in Fig. 8.7. Plouffe et al. [67] exploited the ability of REDV peptide toward selective EC attachment in polydimethylsiloxane microfluidic devices. Microfluidic devices coated with REDV were used for the adhesion-based separation of ECs from heterogeneous suspensions containing ECs, SMCs, and fibroblasts. The adhesion of ECs on REDV-coated devices was significantly higher than the other cell types. Therefore REDV was confirmed to be a selective peptide favoring EC adhesion respect to SMCs. Other studies demonstrated that REDV may hinder the adhesion of platelets [68].

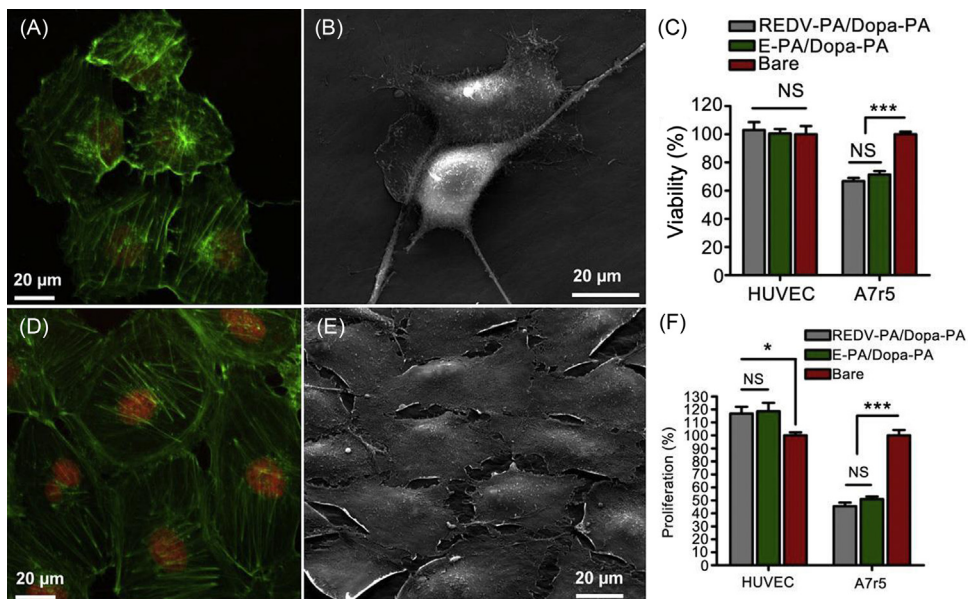


FIGURE 8.7 (A,B,D,E) HUVECs preserved their morphology and formed filamentous actin-based stress fibers after 24 and 72 h on REDV-PA/Dopa-PA network. (C) HUVECs were completely viable on both PA surfaces compared to the bare steel surface. On the contrary, A7r5 rat aortic smooth muscle cells showed decreased viability on coated steel surfaces compared to the bare steel surface. (F) HUVECs proliferation was higher on both PA-coated surfaces while the proliferation of A7r5 cells decreased significantly on the PA networks. *** $P < .0001$, * $P < .05$. Dopa, 3,4-Dihydroxyphenylalanine; HUVECs, human umbilical vein endothelial cells; PA, peptide-based self-assembled. Source: Reprinted from Ceylan H, Tekinay AB, Guler MO. Selective adhesion and growth of vascular endothelial cells on bioactive peptide nanofiber functionalized stainless steel surface. *Biomaterials* 2011;32:8797–805. Available from: <https://doi.org/10.1016/j.biomaterials.2011.08.018>, ©2011, with permission from Elsevier.

Unlike the RGD peptide, which is present in many ECM proteins and binds a large number of cellular integrins, including $\alpha\text{IIb}\beta 3$ on platelets as well as $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ on ECs, CRRETAWAC is a non-natural peptide, identified from a phage display library for its interaction with human $\alpha 5\beta 1$ integrin [69,70]. Larsen et al. [71] functionalized polytetrafluoroethylene (PTFE) vascular grafts with CRRETAWAC. In addition, Meyers et al. [72] used CRRETAWAC as a bioactive stent coating. Dudash et al. [73] reported the cross-reactivity of the cell adhesive peptide CRRETAWAC between human and porcine ECs (hECs and pECs). In this study, they demonstrated that CRRETAWAC peptide is capable of binding pECs specifically, with pECs growing then similarly compared to hECs. *In vitro* validation of the porcine model is critical for ensuring effective validation for *in vivo* testing of CRRETAWAC-coated PTFE vascular grafts.

Concerning scaffolds for cardiac regeneration, functionalization with RGD which is present in several adhesion proteins, such as fibronectin, vitronectin, laminin, and collagen type I, is of crucial relevance. Schussler et al. [74] investigated an *in vitro* method to improve the contractile properties of CMs seeded on a collagen scaffold. In particular, they modified commercially available collagen scaffolds (Avitene Ultrafoam hemostat sheets) with GRGDS by covalent coupling. As shown in Fig. 8.8, results indicated that contractility in cell-seeded collagen scaffolds was significantly improved by the covalent grafting of GRGDS to collagen scaffolds, probable due to the increased availability of ligands for the $\alpha\text{v}\beta 5$, $\alpha\text{v}\beta 3$, and $\alpha 5\beta 1$ integrins. Moreover, the improvement of cell adhesion, survival, growth, and differentiation of CMs in GRGDS scaffolds enhanced mechanical performance of the constructs. Such scaffolds appeared promising for future clinical applications.

Rosellini et al. [75] covalently grafted on poly(ϵ -caprolactone) (PCL) substrates two penta-peptides: GRGDS from fibronectin, and YIGSR (Tyrosine-Isoleucine-Glycine-Serine-Arginine) from laminin. GRGDS peptide was shown to promote the adhesion of C2C12 myoblasts, to stimulate integrin receptors relevant in early cardiac development ($\alpha 5\beta 1$, $\alpha\text{v}\beta 3$) and to promote cell proliferation. On the other hand, YIGSR mainly promoted C2C12 myoblast differentiation, as shown by the appearance of multinucleated myotubes even in the absence of a differentiation medium.

Hayoun-Neeman et al. [76] developed functionalized alginate scaffolds able to induce the differentiation of human embryonic stem-derived CMs in order to obtain functional cardiac tissues. The macroporous alginate scaffolds were modified with two different peptides: RGD and heparin-binding peptide, to mediate cell–matrix interaction by both an integrin-dependent and independent mechanism, respectively. The authors demonstrated that the presence of both peptide types was needed for functional tissue development.

The same approach based on alginate functionalization with RGD peptide was successfully investigated by Shachar et al. [77]. They obtained 3D porous scaffolds through freeze–drying technique using both neat alginate and alginate grafted with RGD via carbodiimide chemistry. They demonstrated that the immobilization of RGD peptide into 3D porous alginate scaffolds enhanced the formation of functional cardiac tissue.

Table 8.2 summarizes relevant examples of scaffolds and implants surface-functionalised with peptide-ligands for different applications, including the cardiovascular field.

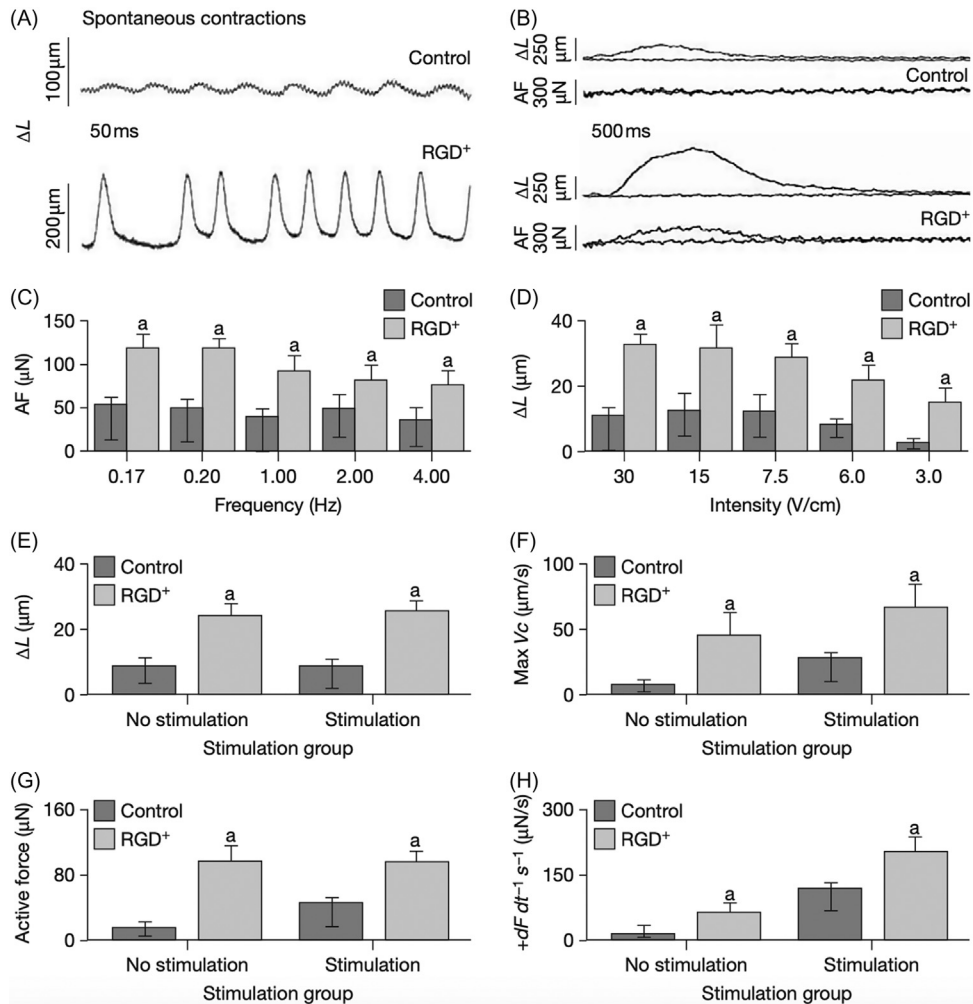


FIGURE 8.8 Contractile performance in GRGDS (RGD+) functionalized and control scaffolds seeded with cardiomyocytes. (A) Muscle shortening length as a function of time. (B) Muscle shortening length and AF of contractions at 0.17 Hz electrical stimulation frequency. (C) Effects of electrical stimulation frequency on AF. (D) Effects of electrical stimulation intensity on maximum extent of muscle shortening (ΔL). (E) Contractile performance expressed as maximum extent of muscle shortening. (F) Contractile performance expressed as maximum shortening velocity (V_c) at preload. (G) Contractile performance expressed as AF. (H) Contractile performance expressed as positive peak of the force derivative ($+dF/dt$). Values are means $\pm$ std. dev., (A) $P < .05$. AF, Active force; RGD, arginine–glycine–aspartic acid GRGDS, glycine–arginine–glycine–aspartic acid–serine–proline. Source: Reprinted from Schussler O, Coirault C, Louis-Tisserand M, Al-Chare W, Oliviero P, Menard C, et al. Use of arginine–glycine–aspartic acid adhesion peptides coupled with a new collagen scaffold to engineer a myocardium-like tissue graft. *Nat Clin Pract Cardiovasc Med*. 2009;6:240–9. Available from: <https://doi.org/10.1038/ncpcardio1451>, ©2009, with permission from Springer Nature.

TABLE 8.2 Widely adopted peptide ligands in the surface functionalization of scaffolds and implants to guide cell behavior.

Peptide sequence	Biological function	Material	Application
<i>General applications</i>			
RGD	Adhesiveness	Ti substrate	Multilayered HA/CH coating with antibacterial and cell adhesive properties [61]
<i>Vascular applications</i>			
GRGDSP	Proliferation and spreading of ECs	Poly(carbonate urethane)s	Reendothelialization of small-diameter vascular grafts [63]
REDV	Selectivity for ECs attachment	Self-assembled functional peptides used as coatings for stainless steel	Reendothelialization of metal vascular implants [66]
REDV	Selectivity for ECs attachment	PDMS microfluidic devices	Adhesion-based separation of ECs from heterogeneous cell suspensions [67]
CRRETAWAC	Selectivity for ECs attachment	PTFE vascular grafts	Reendothelialization of vascular prostheses [71]
CRRETAWAC	Selectivity for ECs attachment	Preliminary coating of culture plates	Reendothelialization of stents [72]
CRRETAWAC (incorporated a FSP)	Selectivity for ECs attachment	Self-assembled monolayers of perfluorosilanes on glass slides	Validation of the ability of CRRETAWAC to bind both porcine and human ECs [73]
<i>Cardiac regeneration</i>			
GRGDS	To increase availability of ligands for the $\alpha_v\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors in cardiomyocytes	Collagen scaffold (Avitene Ultrafoam hemostat sheets)	To improve the contractile properties of cardiomyocytes [74]
GRGDS, YIGSR	To improve cell adhesion, proliferation, and differentiation	PCL-based substrates	C2C12 myoblast attachment, proliferation and differentiation [75]
RGD and HBP	To induce the differentiation of human embryonic stem-derived cardiomyocytes	Alginate scaffolds	To achieve cell–matrix interaction mediated by an integrin-dependent and independent mechanism [76]
RGD	To increase the formation of functional cardiac tissue	Freeze–dried alginate scaffolds	Cardiac regeneration tested using neonatal rat cardiac cells [77]

CH, Chitosan; ECs, endothelial cell; FSP, fluorosurfactant polymer; HA, hyaluronic acid; HBP, heparin-binding peptide; PCL, polycaprolactone; PDMS, polydimethylsiloxane; PTFE, polytetrafluoroethylene; RGD, arginine–glycine–aspartic acid.

8.4 Ligand functionalization of nanoparticles for cell targeting

In the last few decades, nanomedicine has been widely exploited for different applications, including the diagnosis, prevention, and treatment of diseases. NPs can encapsulate different types of molecules (even combined), protect them from degradation, transport them at the target site, and even deliver them to specific cell types. For these reasons, nanocarriers have been extensively employed in cancer diagnosis and treatment [78–80]. However, nanomedicine also covers different therapeutic fields, including the treatment of cardiovascular diseases [10,81].

NP size is in the order of few hundred nanometers or less, allowing them to elude renal clearance [82]. They can be prepared from diverse materials, both organic and inorganic, or even by organic–inorganic combinations [83,84]. Examples of carrier materials for NPs applied in cardiac regenerative medicine include lipids (liposomes) [85,86], polymers such as PEG–PLA (PEG-b-poly(D, L-lactide)) [87] or dextran [88], gold [89,90], and iron oxide [91].

Besides the small size and the versatile composition, the feature that mostly prompted their application in biomedicine is their high surface-to-volume ratio. Their large available surface can be modified and functionalized with molecules able to direct NPs to the target tissue, where they finally deliver their cargo (drugs or other bioactive molecules) exerting the desired medical function. Based on that, NPs can establish new pharmacodynamics and pharmacokinetics of the therapeutic molecules, carrying them to a precise site of action [92].

Particularly, functionalization of NPs is essential to achieve an efficient cell delivery and even an active targeting. By attaching specific ligands, it is possible to direct NPs toward cell receptors (glycoproteins or GAGs–based): as an example, cells in diseased tissues may overexpress surface receptors (disease markers) which may be targeted by NPs for a specific drug delivery. By this approach, NPs may enter the cells via receptor-mediated endocytosis [93].

In the treatment of cardiac diseases, scientific literature offers different examples and strategies for NP functionalization.

Proper surface tailoring of NPs may enhance their biocompatibility properties and ability for cell uptake. For example, Ornelas-Soto et al. [94] covalently modified the surface of mesoporous silica NPs (MSN) with oleic acid (OA) and evaluated NPs internalization by myocardial cells *in vitro*. NPs were first chemically functionalized with (3-aminopropyl) triethoxysilane to expose primary amines which can react with organic acids. In this case, cis-9-octadecenoic acid was added to obtain aminopropyl/OA-modified MSN particles (MSN-OA). These NPs were tested *in vitro* on rat myocardial cell line H9c2, and showed an increased and dose-dependent uptake compared with nonfunctionalized silica particles, with low levels of cytotoxicity when compared with the nonfunctionalized NPs [94].

Another approach to ameliorate NP uptake consists of the tuning of their external electrical charge. Di Mauro et al. [95] prepared calcium phosphate (CaP) NPs for drug delivery to CMs. NPs were produced through biomineralization-inspired one-pot synthesis using citrate as stabilizer and were characterized by a negatively charged surface that can help the CM membrane crossing. Polarized excitable cells (like CMs) were found to have a

selective affinity for negatively charged NPs, which can also facilitate the formation of not-harmful nanopores for NP internalization [96]. In this work, CaP NPs did not show *in vitro* toxicity on HL-1 cell line and primary mouse CMs and did not affect cell functionality, such as Na^+ , Ca^{2+} , and K^+ ionic currents. Moreover, NPs encapsulating synthetic microRNAs (in this case not-mammalian cel-miR-39–3p from *Caenorhabditis elegans*) were systemically injected *in vivo* in mice showing a significant accumulation in the left ventricle [95].

The same CaP NPs loaded with therapeutic peptides were also proposed for heart targeting through inhalation and tested *in vivo* initially in a rodent model of diabetic cardiomyopathy, followed by a porcine large animal model [97].

As an additional strategy, peptide surface functionalization was exploited to promote active targeting, and one of the first works applying this concept on cardiac cells was published by Dvir et al. [98]. The authors exploited the overexpression of angiotensin II type-1 (AT1) receptor by the infarcted heart [99]. PEGylated liposomes with 142 nm average size were prepared and surface functionalized with a short peptide composed by 4 glycine residues (serving as spacer) followed by 8 residues of angiotensin II, the specific ligand for the overexpressed receptor. AT1 binding NPs, labeled with a fluorescent dye, demonstrated to recognize cardiac cells both *in vitro* and *in vivo*. *In vitro*, primary cardiac cells isolated from neonatal rats showed higher uptake of AT1 binding NPs compared to nonspecific NPs bearing a peptide with scrambled aminoacids (52% vs 27%, respectively). NP specificity was confirmed by exposing NPs to the same cells after 48 h in hypoxia conditions (5% O_2): targeted cells percentage increased from 52% to 83%. *In vitro* results are shown in Fig. 8.9. *In vivo*, AT1 binding NPs were systemically injected into mice after induction of myocardial infarction and demonstrated to accumulate mainly in the left

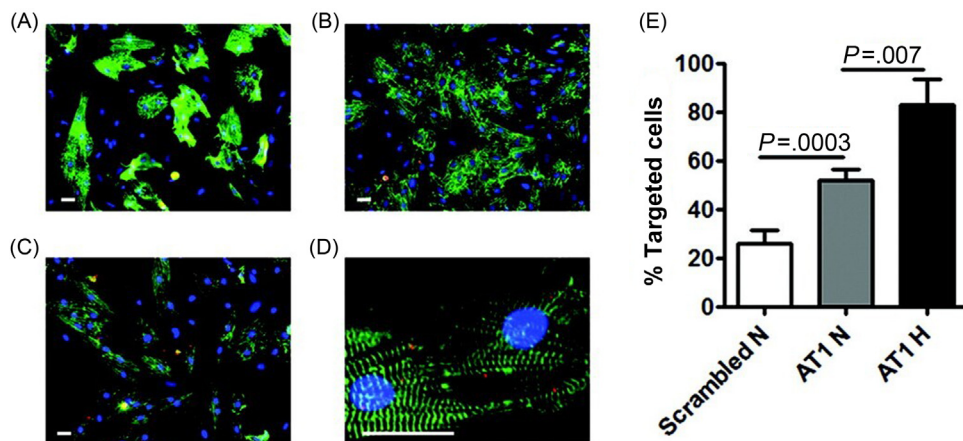


FIGURE 8.9 (A–D) Fluorescent images of *in vitro* cardiac cell-targeting, comparing: (A) targeting with NPs conjugated with nonspecific scrambled peptide; (B) targeting with AT1 binding NPs; (C–D) targeting cardiac cells under hypoxic conditions with AT1-binding NPs. In all panels sarcomeric actinin (green), NPs (red) and nuclei (blue) are shown; Scale bar = 20 μm . (E) Percentage of targeted cells. NP, Nanoparticle. Source: Reprinted with permission from Dvir T, Bauer M, Schroeder A, Tsui JH, Anderson DG, Langer R, et al. Nanoparticles targeting the infarcted heart. *Nano Lett* 2011;11:4411–4. Available from: <https://doi.org/10.1021/nl2025882>. ©2011, American Chemical Society.

myocardium and less in the other organs; they were also administered to healthy mice but they did not accumulate in the heart [98].

In another work, porous silicon (PSi) NPs were functionalized with three different peptides to specifically target cardiac cells. The first peptide was a circulating cardiac hormone (atrial natriuretic peptide, ANP), known for its cardioprotective properties, antiapoptotic action, and its capacity to inhibit hypertrophy; it is also able to bind a receptor expressed in both CMs and cardiac fibroblasts. The other two peptides used (named P2 and P3) were selected with a phage display approach, focused on identifying sequences able to target ischemic myocardium. The three peptides were covalently bonded to the free carboxyl groups by EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide) chemistry. The three different pools of functionalized NPs were tested for biocompatibility and cellular uptake *in vitro* in H9c2 cell line (cardiomyoblast cells), primary CMs, and non-muscle cells to cover all heart cell types. Compared to non-modified NPs, functionalized NPs showed cytocompatibility at concentrations up to 50 µg/mL for all cell types, as shown in Fig. 8.10. On the other hand, the uptake of all the peptide-modified NPs significantly increased in H9c2 cell line, while primary CMs showed high uptake also of negatively charged non-functionalized NPs, probably due to a nonspecific binding. Subsequently, specificity of NPs for cardiac cells was demonstrated *in vivo*: in rat models of isoprenaline-induced infarct, radiolabeled particles were inoculated via tail vein and proved to be accumulated in the heart 10 minutes after administration [100].

The same authors exploited ANP hormone in acetylated dextran (AcDX) NPs carrying two model drugs, CHIR99021 and SB431542, useful in increasing the efficiency of direct reprogramming of fibroblasts into CMs [101,102]. In this work, spermine-modified AcDX was loaded with the hydrophobic drugs using an oil-in-water emulsion procedure to

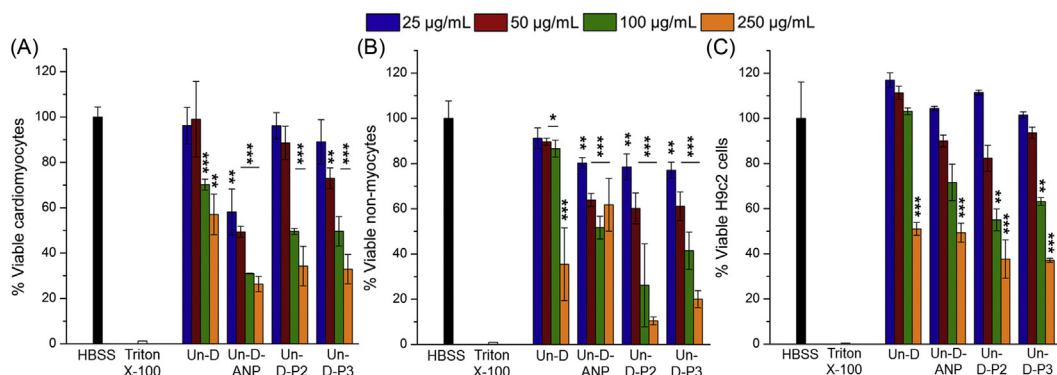


FIGURE 8.10 Cytotoxicity profiles of (A) primary cardiomyocytes, (B) primary non-myocytes, and (C) H9c2 cells after exposure to different concentrations of non-functionalized (Un-D) and peptide-functionalized nanoparticles (Un-D-ANP/P2/P3). All conditions are normalized to the negative control (cell treated with HBSS, pH 7.4). * $P < .05$, ** $P < .01$, and *** $P < .001$. HBSS, hank's balanced salt solution. Source: Reprinted from Ferreira MPA, Ranjan S, Correia AMR, Mäkilä EM, Kinnunen SM, Zhang H, et al. *In vitro* and *in vivo* assessment of heart-homing porous silicon nanoparticles. *Biomaterials* 2016;94:93–104. Available from: <https://doi.org/10.1016/j.biomaterials.2016.03.046>, ©2016, with permission from Elsevier.

produce NPs. Then, their surface was functionalized with PEG and ANP hormone using carbodiimide chemistry.

Functionalized NPs demonstrated *in vitro* cytocompatibility with cardiac fibroblasts even at high concentrations (up to 250 $\mu\text{g/mL}$), while toward CMs they were non-toxic only at low concentrations (up to 25 $\mu\text{g/mL}$). Remarkably, in this work, NPs were allowed to carry two water insoluble drugs; moreover the pH responsiveness of AcDX allowed the triggering of drug release from the endosomes, after NP uptake by the cells [88].

Other peptides selected through phage display technique were applied for targeted heart treatment. One significant example is represented by the short linear peptide CRPPR (cysteine-arginine-proline-proline-arginine), which specifically binds to the heart endothelium [103]: CRPPR peptide was conjugated to phospholipid-based liposomes using a PEG molecule (3600 Da) as spacer to expose the peptide [104]. Liposomes were loaded with a fluorescent dye as model drug and systemically administered *in vivo* in a murine model of myocardial disease (male C57BL/6 mice, 2-month old). Such particles demonstrated to accumulate more in the cardiac ECs compared to the surrounding tissues, and accumulation increased with time after the MI event [105].

Another targeting strategy involves NP functionalization with peptides binding heparan sulfates (HS) in the GAG molecules on the cell surface. Generally, NPs are coupled with cell-penetrating peptides (CPP), known for their capacity of facilitating cell endocytosis of extracellular cargoes without affecting cell viability and proliferation [106]. As an example, in the work published by Osman et al. [107], they utilized this approach to develop a new tool for gene delivery with the prospective to treat genetic disease, such as cystic fibrosis, where a mutation of the *CFTR* gene causes lung failure in the long-term. In this work, DNA NPs were functionalized with a HS-binding sequence, precisely a 16-aminoacid sequence derived from fibroblast growth factor 2, coupled with an amphiphilic sequence (identified as LK15), to help DNA condensation ability and intracellular trafficking, and an octaarginine (8 R) as CPP. The resulting peptide (FLR) was covalently coupled with PEG maleimide chains (5 kDa) via a thioether linkage after addition of a N-terminal cysteine. The resulting cationic peptide facilitated encapsulation of DNA molecules in the NP core, while PEG chains formed a layer on NP surface that helped to inhibit particle aggregation. Fig. 8.11 shows DNA-loaded NP composition and structure.

The PEGylated NPs were tested for their delivery *in vivo* in mice lungs by local administration with an intratracheal microspray apparatus. The DNA used consisted of a plasmid encoding for a luciferase reporter that enables noninvasive gene expression quantification, through bioluminescence measurement. Mice treated with PEG–DNA complexes showed higher transgene expression compared to treatment with DNA alone or combined with a polymer-based vector [107].

GAG-binding peptides can also be combined with magnetic NPs (Nanomag-D dextran shell/iron oxide core), by covalently coupling the peptide through -COOH functional groups with EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)/NHS chemistry. In this case, the GAG-binding peptide was composed of 21 residues coming from heparin-binding epidermal growth factor (HB-EGF) and again by 8-arginine residues. NP uptake was demonstrated *in vitro* in NIH3T3 fibroblast cell line, through light microscopy Prussian blue iron-staining [106].

Table 8.3 summarizes relevant examples of NP functionalization in cardiac regenerative medicine, classified by targeting implementation.

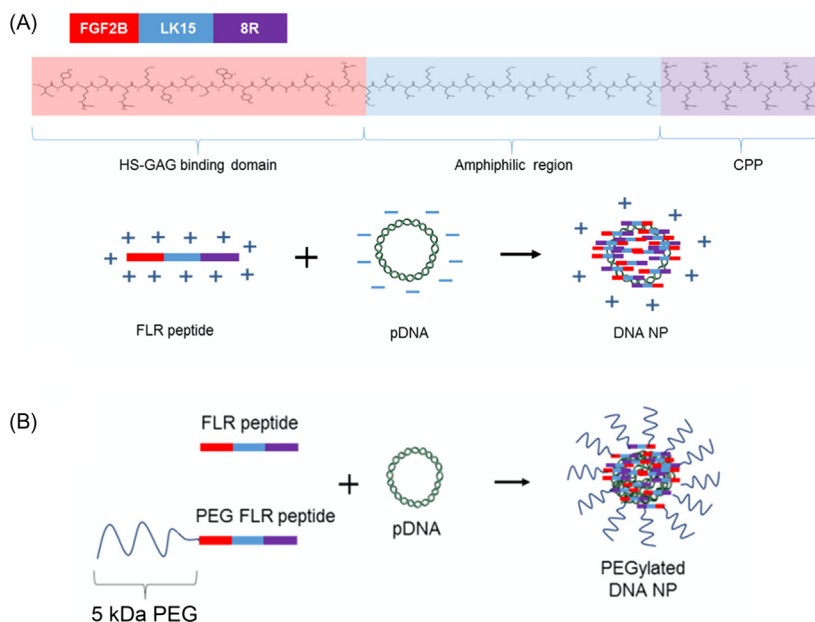


FIGURE 8.11 Schematic representation of DNA-loaded NP structure. (A) FLR sequence, a multidomain peptide composed of a HS GAG-binding domain (red), an amphiphilic region (blue), and CPP (purple). When FLR peptides are mixed with DNA, they establish electrostatic interactions with the phosphate groups (negatively charged) of the plasmid leading to NPs formation through self-assembly. (B) Final NP structure, after PEGylation of FLR sequence. CPP, Cell-penetrating peptide; GAG, glycosaminoglycan; HS, heparan sulfates; NP, nanoparticle. Source: Adapted from Osman G, Rodriguez J, Chan SY, Chisholm J, Duncan G, Kim N, et al. PEGylated enhanced cell penetrating peptide nanoparticles for lung gene therapy. *J Control Release* 2018;285:35–45. Available from: <https://doi.org/10.1016/j.jconrel.2018.07.001>, ©2018, with permission from Elsevier.

To the best of our knowledge, NP decoration with peptides binding HS on the cell surface has not been exploited for cardiac delivery and deserves investigation.

The functionalization methods reported in this section are only a few examples of how nanomaterial design can be tuned to the final application. As already shown in the last examples, many works in the literature report applications of functionalized nanocarriers to treat different types of disorders, from neurodegenerative to chronic infectious diseases, such as Alzheimer's disease [108], hepatic cirrhosis [109], or HIV [110,111], among the others.

The multitude of applications is possible since NPs' interaction with proteins and cells can be controlled by tuning their size, shape, composition, external functionalization, and electrical properties to achieve different aims [112].

8.5 General discussion and conclusion

Bioactive biomaterials can be obtained by biomaterial functionalization with peptides able to specifically modulate their interaction with cells and the host environment (Fig. 8.1). Depending on the therapeutic purpose, hydrogels, scaffolds, or NPs are used in

TABLE 8.3 Examples of nanoparticle surface functionalization classified by target type.

Surface molecule	Application	NP material	Type of functionalization	Molecules loaded/labeling	Reference
<i>NP tailoring for increased biocompatibility and cell uptake</i>					
cis-9-octadecenoic acid	Biocompatible silica-based particles for delivery to myocardial cells (H9c2 cell line)	Mesoporous silica	Functionalization with APTES to obtain amino groups for oleic acid grafting	FITC (staining)	Ornelas-Soto et al. [94]
Calcium phosphate	To release therapeutics to cardiomyocytes	Calcium phosphate	No functionalization was used: the intrinsic negative charge of NPs was exploited to preferentially target polarized cardiomyocytes	MicroRNAs Short therapeutic peptides	Di Mauro et al. [95] Miragoli et al. [97]
<i>Cell targeting by peptide functionalization</i>					
Peptide with 4 glycine residues (spacer) and 8 angiotensin II residues (DRVYIHPF)	To target infarcted heart tissue by binding the AT1 receptor, overexpressed after hypoxia	PEGylated liposomes	Grafting via carbodiimide chemistry	DyLight649 probe (staining)	Dvir et al. [98]
Peptide sequences selected by phage-display approach	To target selectively ischemic heart	Porous silicon	Grafting via carbodiimide chemistry	Alexa488 (staining) ¹¹¹ InCl ₃ (labeling)	Ferreira et al. [100]
ANP	To target infarcted heart tissue by binding to a tissue-specific cardiac receptor	Porous silicon	Grafting via carbodiimide chemistry	Alexa488 (staining) ¹¹¹ InCl ₃ (labeling)	Ferreira et al. [100]
ANP	Direct reprogramming of cardiac fibroblasts	Acetylated dextran	PEG and ANP grafting on NP surface and crosslinking chemistry	SB431542 CHIR99021	Ferreira et al. [88]
Short peptide (CRPPR) identified by phage-display approach	To target heart with efficient trans-endothelial transport	PEGylated liposomes	Liposome preparation using Lipo-PEG-Peptides	Alexa555 (staining) Radioactively labeled lipid, ¹⁸ F-FDP (labeling)	Zhang et al. [105]

(Continued)

TABLE 8.3 (Continued)

Surface molecule	Application	NP material	Type of functionalization	Molecules loaded/labeling	Reference
<i>Cell targeting by polysaccharide functionalization</i>					
Multifunctional FLR peptide (Fig. 8.11)	Target lung cells through HS for gene delivery	Plasmidic DNA	NPs are prepared by complexation through FLR self-assembly (Fig. 8.11)	DNA labeled with Cy3 or Cy5, encoding for firefly luciferase	Osman et al. [107]
Peptide with 21 residues of EGF and 8 Arginine residues	Delivery of molecules for cell labeling or cell targeting	Nanomag-D (Dextran shell/iron oxide core)	Grafting via carbodiimide chemistry	None	Dixon et al. [106]

18F-FDP, 18F-fluorodipalmitin; *ANP*, atrial natriuretic peptide; *APTES*, (3-aminopropyl)triethoxysilane; *AT1*, angiotensin II type-1; *Cy3/5*, cyanine dye 3/5; *EGF*, epidermal growth factor; *FITC*, fluorescein isothiocyanate; *HS*, heparan sulfates; *NP*, nanoparticles; *PEG*, poly(ethylene glycol).

regenerative medicine, and all of them can be functionalized with peptide ligands to achieve specific biological functions.

The functionalization of biomaterials with bioactive peptides (Tables 8.1–8.3) should follow specific general rules to achieve the desired biological functionality. First of all, the bioactivity of the peptides can be enhanced by specific flanking amino acids, which help the peptide to assume a more biomimetic conformation for improved ligand-receptor interaction [69]. Although RGD triplet is the minimal peptide sequence allowing integrin binding, it is generally used in combination with flanking amino acids to improve its effectiveness: as an example, RGDS [28] and CRGDS [35] have been frequently employed, instead of RGD.

Furthermore, spacer units have been widely used to expose bioactive peptides and to increase their conformational degrees of freedom with the final aim to enhance receptor-ligand binding [23,24]. A spacer unit based on ethylene glycol oligomers has been frequently employed to exert an additional antifouling function, avoiding nonspecific protein adsorption, as it could hinder cell interaction with the bioactive peptide [23,24].

Cell adhesion, spreading, and cytoskeletal organization increase as a function of bioactive peptide concentration with a sigmoidal trend whereas, at fixed peptide density, a clustered distribution of the peptide further enhances cell attachment compared to random peptide distribution [69]. On the other hand, cell migration rate has shown a bell-shaped trend as a function of the bioactive peptide concentration [69]. In general, cell binding to adhesive peptides is necessary to generate the forces required for cell migration and to secrete proteolytic enzymes, then leading to progressive degradation into an increasingly porous structure [32].

In conclusion, as a general consideration, peptide chemistry (including flanking amino acids and spacer chains), as well as spatial distribution and density should be regulated to efficiently achieve the target biological functionality of the substrate (hydrogel or scaffold/implant). However, some specific considerations depend on the substrate used for the functionalization.

Biomimetic hydrogels should mimic natural ECM behavior, including a control in the temporal presentation of the bioactive peptides as to regulate ECM deposition by the cells. For instance, this can be obtained by photo-driven removal of a caged group to activate receptor-ligand interactions ([25]; Fig. 8.2) or by enzymatic cleavage of bioactive peptides to decrease cell attachment [27]. MMP-sensitive moieties are also fundamental to achieve cell migration and infiltration within the hydrogels [31]. For this reason, hydrogels have been frequently provided with both adhesive and enzymatically cleavable peptide sequences [33,34]. Furthermore, hydrogel degradation and/or cleavage of adhesive peptides can stimulate cells to produce their own ECM, remodeling the hydrogel [27].

In some cases, hydrogels have been functionalized with combinations of peptides to optimize their biological properties. As an example, PEG-based hydrogels have been grafted with RGDS, VGVAPG and P15, derived from fibronectin, elastin, and collagen-1 respectively, to mimic fibrotic microenvironment, stimulating VIC activation into myofibroblasts [28].

Currently, there is no agreement on the use of adhesive peptides in hydrogels for cell release: a few authors showed they may stimulate immune response, decreasing cell viability [43], while others have demonstrated their positive effect on cell delivery [52].

In addition, antiinflammatory peptides, such as the IL-1RIP peptide sequence [36; Fig. 8.3] or the highly specific TNF- α binding sequence WP9QY [37] may preserve cell viability within cellularized hydrogels.

Literature agrees on the use of adhesive peptides as a tool to stimulate cell recruitment in endogenous regenerative strategies. Finally, hydrogel progressive degradation through functionalization with cleavable peptides is fundamental for their functionality: it causes progressive cell release from hydrogels designed for cell therapy, while it allows hydrogel remodeling by recruited cells in the case of endogenous regeneration strategies.

Specifically, for cardiac regenerative medicine, therapeutic hydrogels may be functionalized with different bioactive peptides (Table 8.1) depending on the final application. In detail, acellular hydrogels for endogenous cardiac regeneration should be functionalized with peptides able to stimulate angiogenesis, cell recruitment, and ventricular function recovery, while cellularized hydrogels should have cardioprotective, pro-survival, and antioxidant properties and additionally be able to promote cardiac phenotype development and maturation of delivered stem cells. A few examples of relevant peptide sequences for therapeutic cardiac hydrogels include: RGD, able to favor cardiac fibroblast recruitment [45] and cell adhesion (Fig. 8.6 [55]; RoY peptide (YPHIDSLGHWRR), stimulating angiogenesis (Fig. 8.5) [46]; glutathione, supporting cell adhesion and protecting cells from oxidative stress [47–49]; QHREDGS, conferring cardioprotective and pro-survival properties, promoting CM attachment and contraction [50–53], as well as myofibroblast recruitment [52]; Jagged1-mimicking peptide, activating early cardiac development, as well as survival and differentiation of CPCs [41,42; Fig. 8.4).

Synthetic polymer scaffolds and implants have been widely used for cardiovascular applications due to their advantageous mechanical properties. However, as they lack ligands for interaction with integrin receptors, they generally need surface functionalization to meet specific biological requirements (Fig. 8.7). Cardiovascular stents or vascular prostheses should stimulate a rapid endothelialization process to avoid thrombosis. For this reason, several authors have proposed surface functionalization with adhesive peptides [63,66,71,72]. However, RGD is not an optimal choice for this application as it also stimulates platelet adhesion and activation. For this reason, the natural REDV and unnatural CRRETAWAC peptides have been proposed to stimulate selective EC attachment versus smooth muscle cells and platelets adhesion [67,73]. Surface functionalization of cardiovascular implants makes use of covalent strategies and benefits from the co-functionalization with antifouling molecules, avoiding unspecific protein absorption, which may lead to thrombus formation.

On the other hand, several types of scaffolds for cardiac regeneration have been prepared from synthetic and natural polymers. In both cases, peptide functionalization has been frequently performed to improve cell adhesion, proliferation, and differentiation (Fig. 8.8). RGD has been one of the most used ligands in the field [76,77]. In addition, more specific peptides have also been proposed depending on the target biological function: particularly, laminin-derived peptides have been demonstrated to stimulate stem cell differentiation into cardiac phenotype [75].

Finally, nanomedicine tools represent a promising approach for the management of cardiac diseases, by directly supplying drugs, hormones, and oligonucleotide-based molecules to targeted cells, with the aim to implement successful new advanced therapeutic

strategies, such as gene therapy and cell reprogramming (Figs. 8.9–8.11). NPs have the advantage of allowing minimally invasive intravenous administration through the systemic circulation, or they can either be delivered locally using injectable gel carriers, avoiding the need for invasive surgical procedures. Another option is the administration through inhalation which allows a rapid translocation of NPs from the pulmonary tree to the bloodstream and to the myocardium, where their cargo can be quickly released [97]. Functionalization with ligands specifically targeting cardiac tissue may reduce systemic toxicity and increase therapeutic outcomes [98].

Table 8.3 collects relevant examples of peptide ligands for targeting of cardiac cells: specific protein receptors of cardiac cells can be selected as the targets for ligand molecules, while heart-specific heparan sulfate functionalities in the proteoglycan receptors could be a new target to investigate. Generally, surface functionalization of NPs with ligands also involves the use of antifouling surface molecules avoiding unspecific protein adsorption.

The continuous progress in nanomedicine through the discovery of new ligands for targeting specific cell populations may reduce the time to the clinical translation of several investigated approaches. Interestingly, precision nanomedicine could potentially allow clinical translation of *in vivo* gene therapies through safer alternatives to viral vectors, which use has been associated with different drawbacks, including immune response, safety issues, and nontargeting properties [113]. In this context, new emerging strategies such as the direct reprogramming of cardiac fibroblasts into CMs [14,15,114] or the stimulation of cell-cycle reentry by CMs [13] could benefit from precision nanomedicine tools.

As a conclusion, peptide ligand type, temporal and spatial distribution, as well as combination with other peptides and/or molecules (e.g., antifouling molecules) strongly affect the biological behavior of medical devices, including nanosized particles, scaffolds, implants, and hydrogels. Proper functionalization may finely tune the substrate biological properties, paving the way to the clinical translation of new emerging approaches, such as gene therapies for cardiac regeneration, and to the design of *in vitro* models of human cardiac tissue for the effective preclinical testing of innovative approaches.

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On the proliferation of cell proliferation tests

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9.1 Introduction

9.1.1 The need and challenge of assessing cell proliferation on biomaterials

In the rapidly evolving and growing fields of tissue engineering and biomaterials there remain a few core concepts repeatedly demonstrated to be integral to the success of any engineered biomaterial. One such property is the biocompatibility or biotolerability of a material. Buddy Ratner, in his most recent definitions of these abstruse yet common terms, defined biocompatibility as “the ability of a material to locally trigger and guide nonfibrotic wound healing, reconstruction, and tissue integration” and biotolerability as “the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reaction.” [1] Intrinsic to these concepts is the ability to assess cellular responses to materials. Arguably, among cellular responses, the most fundamental and widely applied is proliferation of cells in, on, or around an engineered material. This relatively basic measure of the biological performance of a material is an important element to understand cell and tissue behavior, evaluate manufacturing variability, and compare data across experiments, materials, and laboratories [2].

Proliferation measurements are a key assay that can affect the pipeline of product development that has resulted in 66 ongoing or completed clinical trials of tissue engineering-related products between 2011 and 2018 and \$9 billion in sales in 2017 [3]. The extensive financial and time spent on animal experimentation has provided a driving force behind the development of, and increased reliance on, in vitro alternatives to animal testing. In an analogous industry, drug development cost estimates for drug development and preclinical work range from \$561.9 million for central nervous system drugs to \$1403.5 million US for respiratory treatments [4]. Indeed, the development of drug and biomaterial therapies

is time-consuming, resource intensive, and thus, intrinsically risky. As a result, *in vitro* and *in silico* models [5], as well as better sharing of existing data [6], are needed in evaluating novel materials. Cellular proliferation is one of the first biological *in vitro* measures sought in nearly every instance. Yet, this measure is actually nontrivial to obtain. Here, we review how proliferation is measured in biomaterials and tissue engineering and factors to consider for planning, performing, and interpreting these experiments.

Methods for measuring proliferation need to ideally measure viable cell numbers accurately without disturbing or sacrificing the complex tissue or cell population under examination, which in the most challenging experimental conditions, may be from a rare patient sample or a difficult-to-culture cell type. Of course, rigorous, reproducible, and noninvasive cell counting measurements are desired. Delineating methods and terminology around measuring cell proliferation may benefit the biomanufacturing and regenerative medicine research communities by allowing investigators to more closely monitor temporal proliferation trends of cells grown on two-dimensional (2D) and within three-dimensional (3D) biomaterial and decellularized tissue matrices without sacrificing developing tissue for frequent and direct cell measurements.

9.1.2 Cell proliferation versus cell viability

Another prototypical assay performed in the tissue engineering and biomaterial fields is determining the viability of cells. Distinguishing between cell viability and cell proliferation can be a challenge because cell viability is inherent to proliferation. This has led to some authors conflating the two terms. Viability is a measure of whether cells are alive whereas proliferation is a measure of cell division, of which the most direct outcome is an increase in the number of cells. This distinction is important as not every viable cell divides, which is most typified by osteocytes that remain viable for up to 25 years and yet never divide [7]. A hallmark feature of dead and dying (nonviable) cells is the breakdown of cell and nuclear membranes. As a result, many assays used to distinguish viable and nonviable cells are based on this, such as LIVE/DEAD (Thermo-Fisher), cytoplasmic enzyme lactate dehydrogenase (LDH), or transmembrane potential. On the other hand, proliferation assays are typically based on cell metabolic, enzymatic, or nucleic acid replicative pathways. This chapter focuses on proliferation because cell proliferation is arguably harder to trigger than cell viability and is required in almost all envisioned and realized clinical scenarios utilizing tissue engineered or biomaterial devices. As will be seen, measuring cell proliferation is complex and requires a multifaceted approach.

9.2 Methods to measure cell proliferation

The array of methods used to measure proliferation can generally be classified into three categories: metabolism based, nuclei acid based, and others. For example, metabolism-based assays include MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide], MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], and WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]; water soluble tetrazolium. These methods are all based on the ability of cells to

produce some metabolism-related enzymes or products that are detectable. Nuclei acid-based methods, such as PicoGreen and CyQUANT, rely on the intercalation of fluorescent probes with cellular nucleic acids. Other methods are varied and include ATP (adenosine triphosphate) measurements, counting nuclei or immunolabeled cells, flow cytometry, and TEER (transepithelial/transendothelial electrical resistance).

Clearly, a wide range of assays have been developed over the past 50 years to measure cell proliferation. An older estimate [8] (1995–2009) surveying cancer research journals found that the overwhelming majority of published research used MTT assays to measure proliferation, followed by MTS and WST-1. These three methods are all metabolism-based assays with similar chemistries, detailed below. Under 1% of published reports used alamarBlue, PicoGreen, or CyQUANT methods in comparison. A wider variety of assays are now routinely used. Moreover, many authors multiplex their analyses to obtain finer details on cellular proliferation and behavior.

No formal, comprehensive estimates have been made for the usage of different proliferation assays in biomaterials and tissue engineering literature. However, a cursory summary of methods used to measure proliferation in these field is shown in Fig. 9.1 for two historically leading journals in the field, *Biomaterials* (December–October 2018) and *Tissue Engineering Part A* (March 2019–October 2017). The most striking conclusion is the common use of MTT assays in *Biomaterials* and the frequent use of “other” methods in *Tissue Engineering Part A*, such as flow cytometry methods, cell-specific immunofluorescence markers, or clusters of differentiation markers (CD). On the other hand, authors publishing in *Tissue Engineering Part A* preferred DNA (deoxyribonucleic acid) based methods compared to those who publish in *Biomaterials*. An additional conclusion, relating to the overriding philosophies of the biomaterial and tissue engineering fields, is the common use of animal testing in tissue engineering with relatively less in vitro data obtained, compared to biomaterials, before doing animal testing. Indeed, many of the methods for measuring proliferation in *Tissue Engineering Part A* were histological. This likely results from

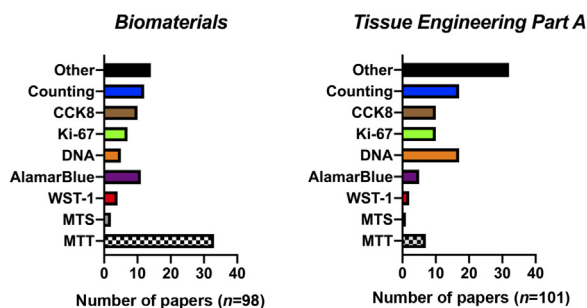


FIGURE 9.1 Number of papers reporting results using specific methods for measuring cell proliferation in *Biomaterials* (December–October 2018) and *Tissue Engineering Part A* (March 2019–October 2017). The counting category includes methods that label cells, such as with a LIVE/DEAD kit or other immunofluorescence methods to count the number of cells in each field of view. The other category includes flow cytometry methods and more specific immunofluorescence markers such as perilipin expression for adipocytes or clusters of differentiation markers (CD).

the need for remodeling and turnover of tissue engineering materials, a process that is difficult to replicate in vitro and not necessary for some biomaterial therapies.

Understanding and interpreting data from all these many measures of a single concept—cell proliferation—can be challenging. We have summarized the most commonly used proliferation methods (metabolic, nuclei acid, and others) below, with an emphasis on the mechanism by which they detect proliferation and confounding variables. We have further gathered experimental considerations for those planning to measure cell proliferation for biomaterials and tissue engineering applications.

9.2.1 Metabolism-based assays

9.2.1.1 MTT

The MTT assay is one of the most popular metabolism-based proliferation assays, and proliferation assay overall. The MTT assay is well studied in comparison to other methods as a result of this popularity. MTT is a colorimetric assay based on a specific tetrazolium salt (first synthesized in the 1950s [9]) and originally applied in the early 1980s for drug screenings [10]. Tetrazolium salts, which are commonly used in proliferation assays, such as others detailed later, are pale yellow substrates that are modified to a dark blue product (formazan) in living cells by various mitochondrial dehydrogenases [11].

The tetrazole ring of tetrazolium salts is disrupted following reduction and transforms from pale yellow into the blue formazan end product. This reduction occurs at the endoplasmic reticulum and, specifically for the MTT assay, within mitochondrial complex II [12]. The positive charge of MTT facilitates cellular uptake because of the plasma membrane potential which results in reduction by nicotinamide adenine dinucleotide (NADH)-dependent oxidoreductases and dehydrogenases [13]. However, each tetrazolium salt tends to have specific preferential locations and/or mechanisms how it is modified. For example, WST-1, another tetrazolium salt detailed later, is primarily reduced at the extracellular surface of the plasma membrane, whereas MTT is reduced by microsomal enzymes and requires reduced pyridine nucleotides as an electron donor [12]. This formazan end product requires solubilization (isopropanol for example) to detect with a plate reader. This is a limitation of the MTT assay as this solubilization step harms viable cells. WST-1 was specifically developed to overcome this limitation [3].

One of the main, if not the most notable, drawback of the MTT assay is that its test results may be influenced by a number of factors, such as human serum albumin [14], vitamin C and vitamin D [15], and flavonoids such as quercetin or rutin [16]. Thiol-containing antioxidant compounds like beta-mercaptoethanol, pyrrolidine-dithiocarbamate, and acetyl-cysteine can reduce MTT tetrazolium salts on their own without the presence of cells [17]. Other materials in media that can influence MTT results include D-glucose [18], glutathione S-transferase [19], and nanoparticles [20].

Another possible drawback to the MTT assay has to do with natural variation in the ability of the cell to reduce MTT. For example, $\text{NAD}^+:\text{NADH}$ ratios fluctuate during a normal mammalian cell cycle (highest in G1 and lowest in S) [21]. As a result, intracellular NADH content will reach its highest around S phase in the cell cycle. Therefore the ability of cells to reduce MTT may be affected and vary based on cell culture conditions.

This highlights the fact that the MTT assay measures a set of enzyme activities, which are related and crucial to cellular metabolism, but are just one measure of cell proliferation. This sentiment is true across the wide range of assays developed to measure proliferation and introduced below.

9.2.1.2 Other tetrazolium salts

WST-1

WST-1, developed by Berridge [22,23], is another tetrazolium salt that is negatively charged and desulfonated with an iodine residue compared to MTT. WST-1 is reduced extracellularly to its soluble formazan by electron transport across the plasma membrane of living cells [24]. This process is not well characterized but involves superoxides and 1-methyl phenazine methosulfates [22]. The formazan product of the reduction of WST-1 is soluble in water and allows the user to skip a solubilization step before quantification compared to MTT. WST-1 is also reported to be more stable than MTT [12].

XTT

XTT, another tetrazolium salt [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] [25], has a negatively charged inner salt and forms a water soluble formazan product, similar to WST-1. XTT has a low ability to move across the plasma membrane compared to WST-1 [12]. XTT can be reduced in the absence of cells by dithiothreitol, mercaptoethanol, reduced glutathione, L-ascorbic acid, and L-cysteine [12,24]. This off-target reduction likely occurs with other tetrazolium salts but has not been tested to date.

MTS

MTS is another tetrazolium salt that has a weakly acidic inner salt [12]. MTS can be influenced by green tea polyphenols because they increase the activity of succinate dehydrogenase [26].

CCK8

Several other tetrazolium salts have been developed similar to WST-1 including WST-8 [27], which is currently being marketed as Cell Counting Kit 8 (Dojindo) [12]. WST-8 has been reported to have higher sensitivity than WST-1, MTS, XTT, and MTT [27].

More detailed information on chemical structures and properties of tetrazolium salts are comprehensively reviewed elsewhere [12].

9.2.2 alamarBlue

alamarBlue is a popular proliferation assay that was originally developed to overcome endpoint assays. Its active ingredient is resazurin (with the addition of other compounds to reduce over-reduction); alamarBlue will be used as synonymous to resazurin in this chapter. Past work has compared resazurin and alamarBlue and showed trivial differences. alamarBlue (with its additional compounds) is hypothesized to generate a fluorescent product slower than resazurin but this has not been experimentally shown [2].

AlamarBlue detects proliferation through reduction by mitochondrial enzymes into resorufin [28]. The location of this reaction is unknown but may occur at the mitochondria, plasma membrane, ribosomes, or in the culture media itself [8]. This reduction process is not as well studied as the one for MTT but diaphorase is the most likely reductive enzyme [29]. Diaphorase is an umbrella term used to describe a number of enzymes including dihydrolipoamide dehydrogenase, quinine oxidoreductase, and flavin reductase [30]. Diaphorases are in all cells and are located either in the mitochondria or cytoplasm. However, other reductases, such as NADH dehydrogenase, may use resazurin as an electron acceptor [29].

AlamarBlue reduction results in a change of nonfluorescent AlamarBlue into a detectable pink fluorescent product. AlamarBlue can be used as an endpoint assay, like MTT, or used to create kinetic estimates of cell proliferation. AlamarBlue has been shown to give an acceptable signal with as few as 80 cells [31].

A potential drawback of using the AlamarBlue assay is that resorufin may be further reduced to a colorless, nonfluorescent hydroresorufin under specific conditions [31]. Fetal bovine serum and serum albumin may cause quenching of the AlamarBlue assay [32] and highly reduced cell culture media can also affect AlamarBlue reduction [31].

9.2.3 Nucleic acid-based assays

9.2.3.1 PicoGreen and CyQUANT

Nucleic acid-based assays are based on a fluorescent signal resulting from the binding of a fluorophore to nucleotides. Two common examples of this include PicoGreen (Thermo-Fisher) and CyQUANT (Thermo-Fisher). These types of assays work well for assessing cell proliferation in 3D cultures because they can be used on lysed cells, which facilitate handling and processing of delicate scaffolds [33]. Additionally, they are highly sensitive; the effective linear range for PicoGreen is as little as 0.025 ng of DNA/mL [34].

PicoGreen is a double-stranded DNA fluorophore and is insensitive to single-stranded DNA [35]. Levels of DNA and ribonucleic acids (RNA) are relatively steady in cells, except during preparation for mitosis, which makes DNA and RNA good targets for measuring cell proliferation [36].

However, according to manufacturer's instructions, PicoGreen is sensitive to sodium chloride and magnesium chloride, bovine serum albumin, phenol, and Triton X-100 levels. Another drawback of PicoGreen is the need to fix or digest cells to release DNA for quantification as PicoGreen cannot enter the cell. Reassuringly, collagenous proteins do not interfere with PicoGreen signals after cell lysis in collagen scaffolds [37].

On the other hand, CyQUANT detects both RNA and DNA but has a stronger fluorescent signal when binding to DNA [8]. CyQUANT generally produces a linear signal from 50 to 50,000 cells per well [38]. CyQUANT requires only one freeze/thaw cycle in order to release nucleic acids without any digestion step compared to PicoGreen.

Although proteins at low concentrations do not interfere with the linearity of CyQUANT fluorescence [38], high protein concentrations (>0.01% v/v Immunoglobulin G or >2% bovine serum albumin) do result in a high background signal, most likely caused by competition with DNA for dye binding sites. As a result, measurements

associated with a proteinous biomaterial, such as a collagen scaffold, may result in false readings. However, typical cell culture media with fetal bovine serum has been reported to be acceptable with proper washing of samples [8].

9.2.3.2 Thymidine analogues

A classic example of proliferation testing is (3H)TdR (tritiated thymidine) whereby excess radiolabeled thymidine is added in cell culture and incorporated into newly synthesized DNA during S phase [39]. This assay is rarely performed nowadays as it involves scintillation counters, autoradiograph development, and is logistically challenging [40].

BrdU (bromodeoxyuridine), an alternative thymidine analog, was developed for detection with an antibody to reduce problems associated with (3H)TdR [41]. However, detection of BrdU is also an endpoint assay like 3HTdR [37]. Duque and Rakic [42] compared the two methods in an in vivo setting using rhesus monkeys cerebral cortices. The authors found large differences in the number of cells labeled, distribution of labeled cells, and labeled cell morphology between the two methods. They suggested these differences, in particular the lower BrdU cell numbers compared to (3H)TdR, were due to decreased cell survival rather than decreased cell proliferation in the BrdU group.

9.2.4 Other methods

9.2.4.1 Adenosine triphosphate

ATP-based assays use luciferin and luciferase to create bioluminescence in the presence of oxygen from ATP, an essential cellular energy unit [43]. ATP can be lysed from cells and then measured using this method. ATP-based assays have approximately 100-fold higher sensitivity than MTS [11] and have been shown to detect ATP released from as little as 20 cells [44].

ATP levels in dead cells quickly reduce after death so ATP contributions from dead cells are negligible, but ATP does increase immediately before cell apoptosis [45]. Some work has shown that ATP is unsuitable for assessing proliferation if materials induce cell hypertrophy, which results in increased mitochondrial area and ATP content [46]. Others have shown that cellular ATP concentration varies throughout the cell cycle, reaching a peak in G2/M phase and a minimum in late G1/early S phase [47].

9.2.4.2 Immunofluorescence markers

Another way to measure proliferation is by probing for proteins expressed at specific points in the cell cycle. Ki-67 is the cell cycle associated protein most commonly assayed for this purpose. It should be noted that Ki-67 does not directly delineate cell cycle phases because it is expressed in G1, S, G2, and M, but is absent in the resting phase, G0 [48]. Ki-67 is typically quantified as a percentage of total cells showing positive staining [49]. Other cell cycle markers include: topoisomerase II alpha, whose expression begins in late S phase and peaks in the G2 and M phases [50]; histone H3, which only becomes phosphorylated during the M phase of the cell cycle [51]; proliferating cell nuclear antigen, whose expression increases during late G1 and peaks during S phase [52]; and cyclin E, which regulates the transition from the G1 to the S phase of the cell cycle [53].

9.2.4.3 Nuclei counting

One of the simplest methods for measuring cell proliferation, and arguably a gold standard because of its directness, is counting nuclei previously labeled with, for instance, DAPI (4',6-diamidino-2-phenylindole) or Hoechst 33342. Alternatively, a viability dye such as LIVE/DEAD could be performed and quantified similarly by counting the number of fluorescently labeled cells. Others have chemically fixed cells and then performed scanning electron microscopy after dehydration [54].

9.2.4.4 Hemacytometer

Another simple method to measure proliferation is to trypsinize cells off the test material and count them in a hemacytometer. While simple, this method may be challenging or impossible for 3D scaffolds and has been heavily criticized for its relatively low accuracy and tediousness [55].

9.2.4.5 Transepithelial/transendothelial electrical resistance

TEER is a sensitive measure of the integrity and permeability of cell monolayers [56]. This method operates by first growing cells on permeable filters. Then, two electrodes are placed above and below the filter to apply a current over the cell layer with an additional two electrodes to measure the resulting voltage drop. Electrical resistance is calculated to indirectly measure proliferation [57]. One benefit of this method is constant monitoring of the system and easy acquisition of high resolution growth curves. However, this method has been criticized for its ability to actually detect drug cytotoxicity and requires relatively specialized equipment [56].

9.2.4.6 Flow cytometry

Flow cytometry has been used to measure cell proliferation in multiple applications. The specific and unique technological and methodological characteristics of counting cells using flow cytometry is a very extensive topic that is beyond the scope of this chapter and has been comprehensively reviewed elsewhere [40,58].

A summary of the advantages and disadvantages of the outlined techniques is in Table 9.1.

9.3 Comparison of proliferation tests

A number of comparative studies between different assays have been reported over the years, most often in the drug development and pharmacology literature. Few comparisons have centered on biomaterial or tissue engineering applications. Nevertheless, these previous comparisons of different techniques can provide useful information for designing and interpreting experiments in different fields. Here, we have summarized the results of a select number of these manuscripts that each demonstrate the relevance of considering an important specific factor in the experimental process.

CyQUANT, PicoGreen, and alamarBlue have been compared using a human ovarian carcinoma cell line (OV-MZ-6), epithelial serous ovarian adenocarcinoma cell line (SKOV-3), human umbilical cord perivascular cells (HUCPVC), and human bone marrow-derived

TABLE 9.1 Comparison of different methods used to assess cell proliferation.

Type of method	Advantages	Disadvantages	Does it measure a number of cells directly?	Reference
<i>Metabolism</i>				
MTT	Most commonly used method	Dye needs solubilized with nonaqueous solvent; endpoint assay	No	[2]
WST-1	Does not require solubilizing the dye like MTT	May be influenced by metabolic environment of cell	No	[24]
XTT	Does not require solubilizing the dye like MTT	May result in a signal without the presence of cells	No	[12]
CCK8	Higher reported sensitivity than WST-1, MTS, XTT, and MTT	Exact chemical mechanism is poorly understood	No	[12]
MTS	Does not require solubilizing the dye like MTT	Can be affected by green tea polyphenols	No	[26]
alamarBlue	Low cost	Fetal bovine serum and serum albumin may cause quenching	No	[28]
<i>Nucleic acid</i>				
PicoGreen	Insensitive to single-stranded DNA; effective linear range is as small as 0.025 ng of DNA/mL	Sensitive to sodium chloride, magnesium chloride, and other materials like bovine serum albumin	No	[35]
CyQUANT	Requires only one freeze/thaw cycle compared to PicoGreen	Detects both RNA and DNA; high background protein levels can interfere	No	[8]
Thymidine analogues	Reported strong linear correlation with increasing number of cells	Requires equipment and training for radioactivity work	No	[39]
<i>Other</i>				
ATP	Approximately 100-fold higher sensitivity than MTS	ATP levels can be increased in dying cells	No	[11]
Immunofluorescence	Works well for tissue sections and histology	Time-intensive	Sometimes	[40]

(Continued)

TABLE 9.1 (Continued)

Type of method	Advantages	Disadvantages	Does it measure a number of cells directly?	Reference
Nuclei counting	Low cost; easy to perform	Time-intensive and 3D scaffold may require nontrivial imaging modalities	Yes	[34]
Hemocytometer	Low cost; easy to perform; basic equipment required	High error; user-to-user variability	Yes	[55]
TEER	Can provide continuous data	Does not accommodate all experimental setups; requires special equipment	No	[56]
Flow cytometry	Single-cell resolution	Cells need to be placed in solution	Yes	[58]

ATP, Adenosine triphosphate, TEER, transepithelial/transendothelial electrical resistance.

mesenchymal stem cells (bmMSCs) at 1–4 days of proliferation [8]. The authors showed that alamarBlue overestimated the proliferative activity of the four cell types by 21%–64% compared to PicoGreen and CyQUANT. In addition, the estimation of cell proliferation based on alamarBlue had a poor correlation with CyQUANT ($R^2 = 0.27$) and for PicoGreen ($R^2 = 0.32$) for bmMSCs for reasons not speculated on. PicoGreen and CyQUANT had high correlation values ($0.82 < R^2 < 0.99$) when compared to each other at each time point. Differences between the three examined methods were most noticeable at 4 days when CyQUANT and PicoGreen determined SKOV-3 had the highest proliferation while alamarBlue suggested that OV-MZ-6 had the highest proliferation. These results underscore the fundamental point that, while two proliferation assays may measure two different biomolecular phenomena that contribute to proliferation, there may be differences in proliferation between the two (or more) assays. However, test multiplexing gives greater insight into cell behavior and has further potential utility, such as use in hypothesizing mechanisms of action for drugs [59].

Past work has compared alamarBlue and MTT assays in a high throughput format with a human hepatoma cell line (HepG2) after exposure to 117 drug candidates [13]. The majority of candidate compounds performed consistently across both assays. The authors reported a Z'-factor of 0.85 and 0.82 for alamarBlue and MTT assays, respectively. Z' is a statistical measure used for high throughput screenings and values over 0.80 suggest high quality results for both techniques [60]. However, drugs known to modulate reductases affected both alamarBlue and MTT results, reinforcing that drugs or materials potentially affecting these pathways should not be used with these techniques. For example, cell proliferation on biomaterials that alter the oxidative environment, such as pullulan-based materials [61], may be better determined with assays that do not involve oxidation/reduction.

MTT solubilized into surfactants, either sodium dodecyl sulfate (SDS) or the nonionic polyoxyethylene NP40, has been compared to WST-1 and resazurin on mouse whole spleen cells (Fig. 9.2) [62]. The authors first tested each technique against a range of cell

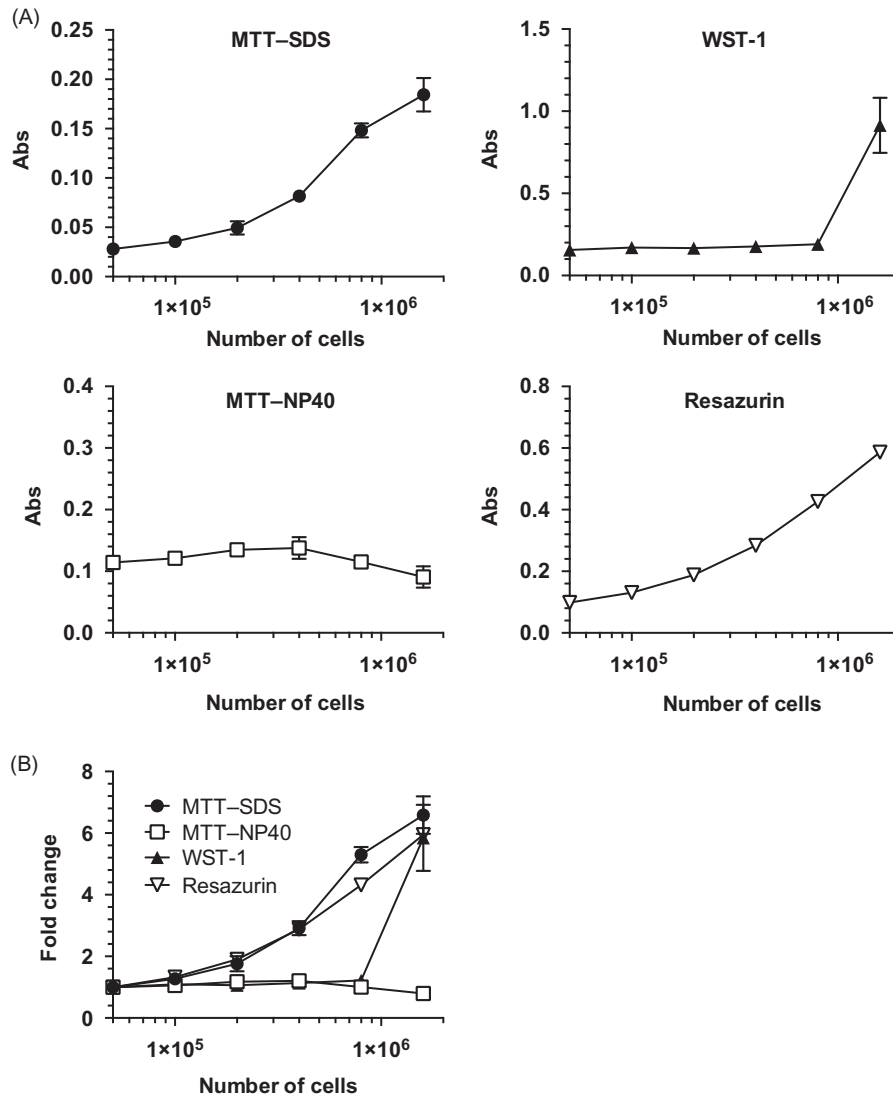


FIGURE 9.2 (A) A serial dilution of mouse whole spleen cells was seeded and assessed for proliferation with MTT (two different surfactant; SDS and NP40), WST-1, and resazurin. (B) The figures in (A) were normalized to the respective minimum values and combined into the same graph to compare their proliferation fold change. SDS, Sodium dodecyl sulfate. Source: Reprinted and adapted from Koyanagi M, Kawakabe S, Arimura Y. A comparative study of colorimetric cell proliferation assays in immune cells. *Cytotechnology* 2016;68(4):1489–98. <https://doi.org/10.1007/s10616-015-9909-2> with permission from Springer.

concentrations to see a dose-response specific for each proliferation test. MTT–SDS and resazurin proportionally increased with more cells, whereas MTT–NP40 showed only a marginal increase. WST-1 did not increase for a wide range of cell concentrations until the highest concentrations. The authors finally stimulated the proliferation of their cells and compared techniques. MTT–SDS and resazurin showed a clear increase in response to an increased amount of stimulant. However, MTT–NP40 only showed a mild increase whereas WST-1 signal did not increase, unless with high concentrations of stimulants, unlike other reagents. Thus, the surfactant selected for solubilizing the dye may influence MTT and similar assays results.

Additional work has compared proliferation of a transfected breast cancer cell line (MCF-7) using ATP, a manual hemacytometer, and nuclei counting [63]. When compared to nuclei counting, the hemacytometer method and ATP assay had an R^2 of 0.93 and 0.90, respectively. High seeding densities increased the error in the hemacytometer method. Alternatively, automatic cell counters were suggested in order to combat error with a manual hemocytometer but these are likely an unnecessary expense for measuring proliferation alone [64].

9.4 Special challenges and experimental design considerations

Proliferation assays may be relatively easy to perform from a technical standpoint but they still require careful planning, such as selecting a proper cell seeding density. This planning becomes more difficult when measuring proliferation in 3D with difficult-to-access embedded cells. Unexpected outcomes occasionally occur, such as when bioactive materials cause false positives and create the need for better controls. Here, we survey a few experimental design and interpretation challenges in measuring cell proliferation.

9.4.1 Cell seeding and proliferation in three-dimensional scaffolds

3D scaffolds pose a unique challenge for measuring proliferation compared to nominally flat surfaces such as ceramics or metals. The most important factor to consider with 3D scaffolds is seeding. This was thoroughly investigated by seeding cells onto the top of four representative scaffolds from across the tissue engineering discipline [65]. Cell seeding efficiency (CSE)—the fraction of initially seeded cells that attach to the scaffold—was calculated using four formulae based on results using PicoGreen. The authors also investigated different freeze-thaw cycles for DNA extraction, cell density, and volume of seeding medium to free scaffold volume ratio. CSE values ranged from 59% to 79% showing that calculation methods affected CSE. The authors also showed that the number of freeze-thaw cycles did affect DNA output and determined that three cycles were optimal for maximum DNA output without extra effort. Indeed, an evidence-based approach is needed to determine relatively basic, yet critical, parameters relating to cell seeding on 3D scaffolds.

Different seeding methods for 3D scaffolds can not only affect CSE but also the distribution of cells within and on a scaffold. Cell seeding has been investigated in poly(lactide-co-glycolide) (PLGA) scaffolds by using static surface seeding, cell seeding with an orbital

shaker, cell suspension injection, and cell seeding with a centrifuge [66]. MTS assay was used for cell counting from cross sections to determine the differences between different seeding methods. Seeding efficiency was 39%–62% depending on the technique. Surface, injection, and orbital seeding methods resulted in around 25% cell death after 3 hours of seeding. Centrifuge seeding method led to around 50% cell death. Surface seeding showed, unsurprisingly, most cells at the surface of the material. Injection seeding resulted in a dense cell presence near the injection point. Centrifuge seeding resulted in biased cell seeding based on orientation in the centrifuge. Orbital shaking showed relatively even seeding throughout the bulk of the PLGA. Overall, these results reinforced the fact that cell seeding affects cell distribution in scaffolds. Finally, it bears reminding that colorimetric assays (the majority of proliferation methods) rely on supernatant analysis. The supernatant is an overall average for the scaffold and does not reveal cell distribution which may be important to specific applications [33].

Investigators extracting DNA from 3D constructs in order to measure proliferation typically use Triton-X or SDS [67]. However, 3D constructs are typically prepared for gene expression using guanidium thiocyanate-based methods to recover RNA. This necessitates two different extraction protocols (DNA and RNA separately) and so an increased sample size. This can be a logistical and resource challenge. Some past work has tested a number of extraction methods and found that RLT (QIAGEN) and RA1 (Macherey-Nagel) buffers simultaneously extracted both DNA and RNA without compromising data integrity of either gene expression or proliferation [68]. As a result, a single extraction can be performed on one sample and result in less labor-intensive, time-consuming methods of extracting both DNA and RNA using some materials such as RLT and RA1.

9.4.2 Cell density

The initial cell seeding or plating density is a fundamental question when measuring proliferation. Adding too many cells can lead to confluence at later time points and associated cell changes while too few cells may be difficult to detect. Uzarski et al. [2] elegantly demonstrated key factors for the use of alamarBlue in 3D scaffolds when a large number of cells is eventually reached (Fig. 9.3). The authors took proximal tubule-derived epithelial cells (RPTE), distal tubule-derived epithelial cells (MDCK), or renal fibroblasts (TK188) and placed them in a recellularized kidney scaffold in a bioreactor. The authors showed nominal differences between using resazurin and alamarBlue at time points from 0 to 9 hours. However, longer incubation times (over 4 hours) resulted in a loss of fluorescence intensity linearity at high cells:volume ratios. The authors also showed that hemocytometers and resazurin did not correlate cell proliferation values at later time points (past 4 days), suggesting a limitation of resazurin once a certain cell density is reached. This reiterates the need for a carefully selected initial cell seeding density. The authors suggested that dimensional constraints imposed by the culture environment, such as the scaffold, may result in morphological changes; that is, a reduction in cell size and a resulting reduction in metabolic activity per cell. As a result, morphological changes in cells may limit the direct correlation of alamarBlue values with overall cell number when using standard curves that were generated from cells grown on tissue culture polystyrene [69].

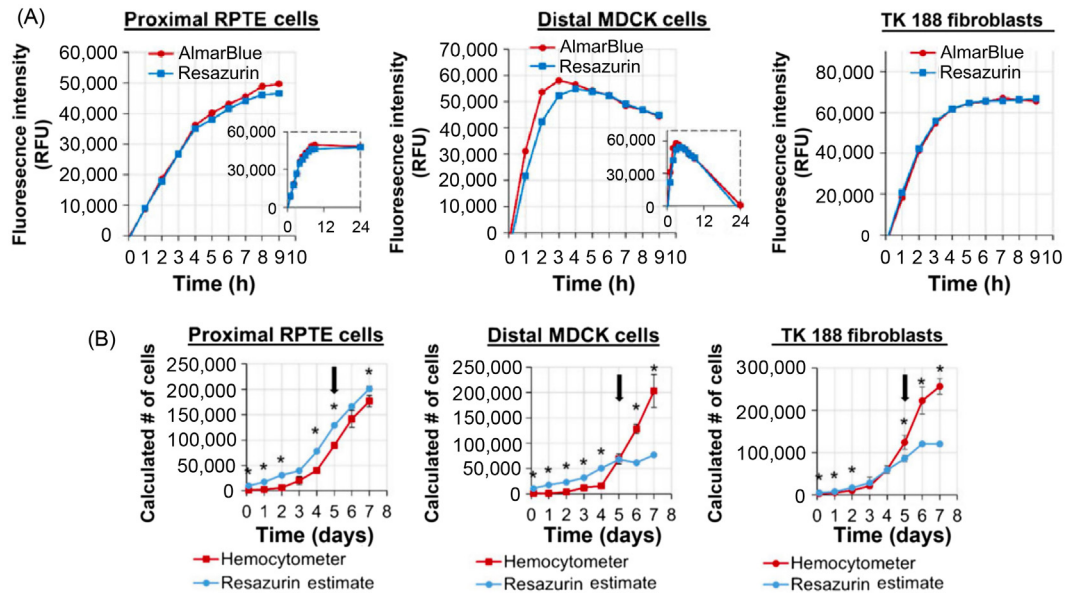


FIGURE 9.3 (A) Comparison of proximal tubule-derived epithelial cells (RPTE), distal tubule-derived epithelial cells (MDCK), and renal fibroblasts (TK188) seeded onto kidney-derived extracellular matrix scaffolds using alamarBlue and resazurin up to 10 h. (B) Comparison between RPTE, MDCK, and TK188 cells using hemocytometer counting and Resazurin up to 8 days. Source: Reprinted by permission from Elsevier: Uzarski JS, DiVito MD, Wertheim JA, Miller WM. Essential design considerations for the resazurin reduction assay to noninvasively quantify cell expansion within perfused extracellular matrix scaffolds. *Biomaterials* 2017;129(847):163–75. <https://doi.org/10.1016/j.biomaterials.2017.02.015>.

9.4.3 Bioactive materials

Some tissue engineered or biomaterial substrates may interfere with proliferation assays. Special care must be taken when working with hydroxyapatite (HAp)-based materials, which are popular for their application in mineralized tissue regeneration. Cells generally proliferate when exposed to HAp. However, some reports have described poor cellular proliferation or cytotoxicity with these materials [70]. This may be due to the ability of HAp to sequester Ca^{2+} , Mg^{2+} , and HPO_4^{3-} from cell culture media, which is exasperated by the high surface area to volume ratio of some of these materials. This phenomenon has been systematically investigated with MTT assays and a correction has been developed for ISO standards based on intense adsorption of ions being responsible for reduced cell proliferation and not presumed release of toxic substances [71]. Some authors [72] have observed similar problems when using MTT and XTT to investigate proliferation on magnesium-based materials. Indeed, cell-free control scaffolds have been shown to induce false positive activity with both MTT and XTT [73]. As a result, additional controls may be necessary to ensure the material has effects on the cells but not on the proliferation assay chemistry itself.

9.4.4 Controls

A final practical consideration for many experiments is the use of a positive control. An appropriate control is tissue culture polystyrene seeded at an equal cell:surface area ratio as the test materials for many materials [74]. Other positive controls may be appropriate. For example, when testing titanium surface functionalization, a plain titanium disk may function well [75–78]. All experimental material groups should be assayed together to enhance biological reproducibility. For example, a single cell passage can affect cell doubling times by over 100% in some instances (murine bone marrow stromal cells) [79].

9.5 Conclusion

A large number of assays have been developed to measure proliferation and are used to determine in vitro biological performance of biomaterials and scaffolds for tissue engineering. We have shown that each of these methods are based on different mechanisms and susceptible to a number of confounding variables and specific challenges for these applications. As a result, cell proliferation experiments should be appropriately selected and well-designed to assess the desired outcomes and interpreted with rigor.

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In vivo models for biomaterials: applications from cardiovascular tissue engineering

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Abbreviations

CSCs	cardiac stromal cells
EC	endothelial cells
ECM	extracellular matrix
EDV	end-diastolic volume
ELPs	elastin-like polypeptides
ESV	end-systolic volume
FDA	Food & Drug Administration
MeHA	methacrylated hyaluronic acid
MI	myocardial infarction
PCL	polycaprolactone
PDLLA	poly-D,L-lactic acid
PEO	polyethylene oxide
PGA	polyglycolic acid
PHB	polyhydroxybutyrate
PLA	polylactic acid
PLGA	polylactide-coglycolide
PLLA	poly-L-lactic acid
PPF	polypropylene fumarate

RLPs	resilin-like polypeptides
SMC	smooth muscle cells
VT	ventricular tachycardia

10.1 Introduction

The cardiovascular system includes the heart and vascular network which delivers blood and nutrients throughout the body and maintains homeostasis [1,2]. Thus, the heart and blood vessels share a common functional process [3]. Heart disease is the leading cause of death for both men and women and was estimated to result in 610,000 annual deaths in the United States alone in 2009 [4]. The search for treatments of cardiovascular disease has led to the development of various biomaterials. [5–7]. Biomaterials have been incorporated in medical therapeutics ranging from gene and drug delivery systems to scaffolds for tissue constructs [8]. Furthermore, recent studies have shown that biomaterial use holds tremendous promise as a novel treatment method for cardiovascular disease [5]. To properly assess the integrity and functionality of these materials, *in vivo* studies that involve implanting and monitoring the product in a living organism are required. These studies are critical to understand the host reaction to the material, the material integration with the surrounding tissue, and the degree of material biocompatibility. Material biocompatibility has always been a concern for implementation of the biomaterial in application. There are currently multiple views of what defines biocompatibility [6,8–10]. However, a general outlook is that biocompatible materials are defined by a lack of toxicity, immunological rejection, or injury to surrounding tissue [8]. The International Union of Pure and Applied Chemistry has defined biocompatibility as “the ability of a material to perform with an appropriate host response in a specific application” [9,10]. The requirements for biocompatibility vary depending on the tissue and are often based on the material characteristics and application [8]. Biomaterials used in vasculature tissue engineering often have a mixture of properties able to mimic the elastic and rigid qualities needed in arterial and venous pressure. In cardiac tissue engineering, biomaterials and constructs are designed to withstand the unique native environment and physical contortions of the heart [6]. This chapter outlines the progress made in testing the performance of biomaterials incorporated within cardiac and vascular tissues using animal models. The review will begin by discussing the specific biocompatibility requirements of materials for cardiovascular tissue. The chapter will then elaborate on the *in vivo* models that have been used to assess biocompatibility with respect to the Food & Drug Administration (FDA) guidelines. Finally, consideration will be given to the applications and future directions of these models.

10.2 Constructs and biomaterials used in cardiac tissue engineering

Cardiac tissue provides a unique challenge in biomaterial engineering due to the structure and functional role of the heart in organisms [11]. Cardiac tissue consists of single nucleated muscle cells, also known as cardiac myocytes, an inner lining of endothelial cells (EC), connective tissue, and in some areas smooth muscle cells (SMC) [12]. Cardiac myocytes exhibit autorhythmicity or spontaneous contraction and depend on gap-junctions

within the cells to propagate action potentials [12–14]. Cardiac monocytes use a calcium ion influx to drive these action potentials resulting in the spontaneous beating observed by cardiac tissue [12,15,16]. This impulse propagation causes a contortion in the tissue's surface [12]. Thus, biomaterials localized within cardiac tissue must contain properties allowing for enhanced adherence, flexibility, and should refrain from interfering with action potentials [5,6]. An optimal biomaterial for cardiac tissue should reduce scarring, increase local cell viability, and vascularization while not causing arrhythmogenesis [6,11]. Arrhythmogenesis is the growth of an irregular cardiac rhythm due to an abnormal pattern or flow of cardiac cell polarization [17]. This condition is a well-characterized symptom of irregular ion transport in cardiac myocytes [17,18]. Arrhythmogenesis has been seen in long QT disorder where sodium or potassium channels are dysfunctional [18] and in electrolyte disorders [17]. Cardiac tissue scarring is often a consequence of myocardial infarction (MI) or heart attacks, which result in dead cardiac tissue [19]. This tissue is cleared by the immune system and replaced with fibroblasts, resulting in scar tissue formation [19]. Further, cardiac tissue scarring post MI has been associated with arrhythmogenesis through electrocardiogram quantification [20] and cardiac resynchronization therapy [21]. A cardiac tissue specialized biomaterial should have sufficient adherence, flexibility, integrity, and porosity to withstand the continual contortions of the heart [6,11].

10.2.1 Materials for cell delivery to cardiac tissue

Cardiac tissue engineering constructs commonly include some combination of biomaterial scaffolds, stem cells, and signaling molecules (Fig. 10.1). Biomaterial injections, both with and without stem cells, have been used as a therapeutic treatment for MI. Injectable biomaterials have become increasingly popular over the past decade and have had productive in vivo outcomes. These outcomes include a reduction of the size of the infarct post MI, an increase in neovascularization, and improvements to general cardiac function [23,24]. As biomaterial injections have evolved, the in vivo assessments have shifted from small animal models to large animal preclinical models [25–27], and clinical trials [28]. In recent developments, biomaterials from alginate and various extracellular matrix (ECM) components (Table 10.1) have been implanted on the myocardium percutaneously [24,25,52,53]. Biomaterial injections and tissue patch implants have improved the negative symptoms associated with cardiac infarction [23,24,26]. In one study, a recent biomaterial of interest, methacrylated hyaluronic acid (MeHA), had positive results in a clinically relevant MI ovine model [26]. In particular, intramyocardial injections of the MeHA hydrogel with varying moduli were used to measure the importance of hydrogel mechanics in therapeutics for MI [26]. The results from the study showed that both low- and high-modulus hydrogels led to an increase in wall thickness of the apex and basilar cardiac infarcts in comparison to controls [26]. However, the MeHA with a high-modulus resulted in improved tissue repair and an infarct area with a significantly smaller area [26]. Further, the high-modulus group showed a reduction in the end-diastolic volume (EDV) and end-systolic volume (ESV) [26]. The high modulus further correlated with superior cardiac output and ejection fraction in comparison to the low-modulus MeHA and control groups [26]. Another study observed the effect of an injectable calcium-cross-linked

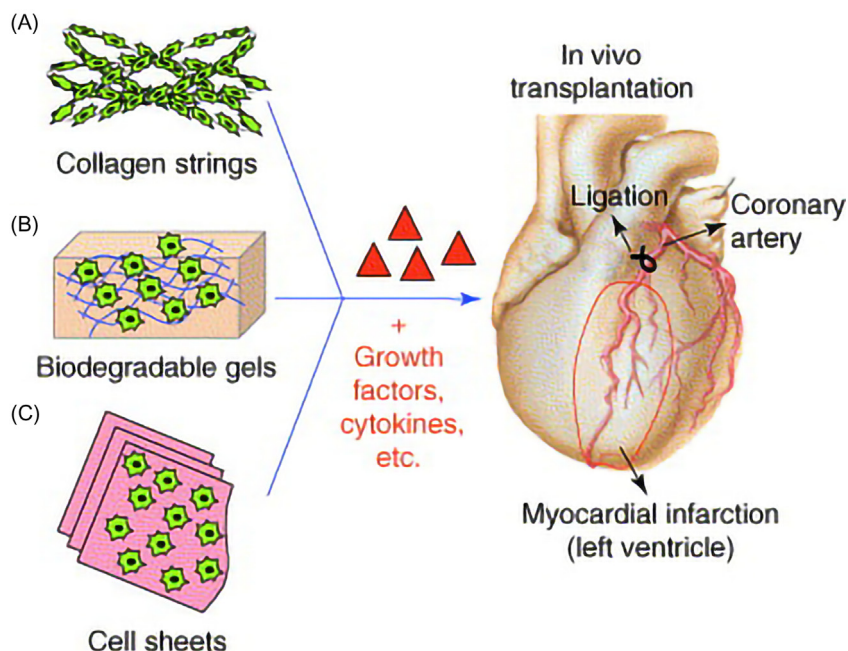


FIGURE 10.1 Schematic of current strategies for cardiac tissue engineering using (A) injectable biomaterials, (B) biodegradable cell-laden gels, and (C) cardiac cell sheets. The incorporation of growth factors and/or cytokines (triangles) have shown to play an influential role in supporting cell differentiation, engraftment and survival, both within the scaffolds and in vivo. Source: Reprinted from Zammaretti P, Jaconi M. Cardiac tissue engineering: regeneration of the wounded heart. *Curr Opin Biotechnol* 2004;15(5):430–4 [22] with permission from Elsevier.

TABLE 10.1 List of natural polymers and derivatives used in cardiac and vascular tissue engineering along with their sources.

Polymer	Source	References
Collagen	Connective tissue (tendons and ligaments)	[29–31]
Hyaluronic acid	ECM of mammals followed by fermentation	[32–34]
Fibrinogen-fibrin	Purified from plasma	[35–37]
Gelatin	Connective tissue (hydrolyzed collagen)	[38–40]
Gelatin methacryloyl	Connective tissue (hydrolyzed collagen)	[41,42]
Chitosan	Exoskeleton of crustaceans (deacetylated chitin)	[43–45]
Matrigel	Secreted by mouse sarcoma cells	[46,47]
Alginate	Found in cell wall of brown seaweed	[47–49]
RLP/ELP	Found in ECM	[50,51]

ECM, Extracellular matrix; ELP, elastin-like polypeptide; RLP, resilin-like polypeptide.

alginate hydrogel in an anterior MI rat-model [54]. This is one of the first results of an in situ model showing successful prevention of cardiac remodeling in regions bearing successive MI in rats. [54]. Serial echocardiography was used 60 days post injection and revealed that early MI treatment, 7 days post infarct, led to an improved EDV and ESV [54]. Moreover, injection into rats aged 60 days post infarction led to similar EDV and ESV results but increased scar thickness [54]. This acellular strategy is appealing because of the well-characterized biocompatibility properties of alginate [55–58]. Other studies have looked at the benefits of producing injectable hydrogel solutions composed of pericardial matrix for cardiac tissue engineering [27,59]. This approach reflects the benefit of using an autologous source for biomaterials which reduces potential immunogenic complications [27]. Injectable biomaterials used as a therapeutic treatment post MI have shown considerable potential; however, further investigation into the long-term effects of these implants is necessary.

10.2.2 Cardiac tissue patches

Constructs including scaffolded cardiac tissue patches have been used to introduce cardiac tissue in post MI in vivo models. The development of patches to repair cardiac tissue can be advantageous for maintaining cell retention in the damaged region [60]. The overall vision for the cardiac patch is that of a cell-based sheet that will be retained and eventually integrated once placed on the infarcted region of the epicardium (Fig. 10.2) [61]. In this way, the patch would reinstate the electrical signals across the necrotic tissue and resume the contractile performance of the heart [61]. This overall therapeutic strategy has led to a variety of cell-based patch designs both with and without the use of biomaterials [60–69]. Fig. 10.3 shows the production of a stem-cell tissue patch using a microextrusion 3D-bioprinting method [70]. One group developed a cardiac tissue patch with clinically relevant dimensions (4 cm × 4 cm) using a matrigel and fibrinogen cell-seeded hydrogel in a polydimethyl siloxane mold [71]. Another team developed 1–4 mm thick heart tissues by molding heart cells with collagen and matrigel and subjecting the tissue to mechanical strain [72]. After implantation in rats, the engineered heart tissue was engrafted and may be a promising therapeutic strategy, but questions remain about the immunogenicity of the constructs [72]. Overall, the most common biomaterials used in scaffolds for cardiac

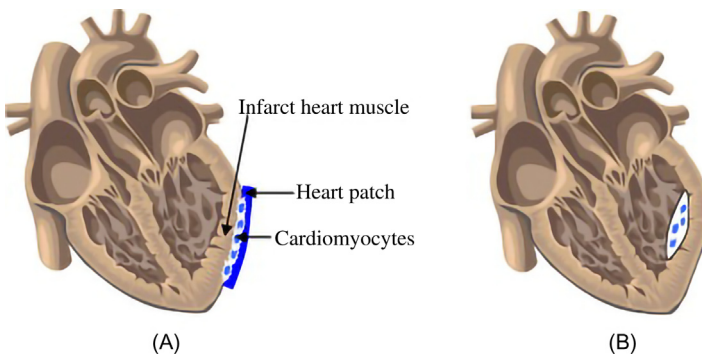


FIGURE 10.2 Schematic illustrations of (A) epicardial and (B) endoventricular heart patch approaches to deliver isolated cells to the infarct regions. Source: Reprinted from Chen Q-Z, Harding SE, Ali NN, Lyon AR, Boccaccini AR. Biomaterials in cardiac tissue engineering: ten years of research survey. *Mater Sci Eng: R: Rep.* 2008;59 (1–6):1–37 [6] with permission from Elsevier.

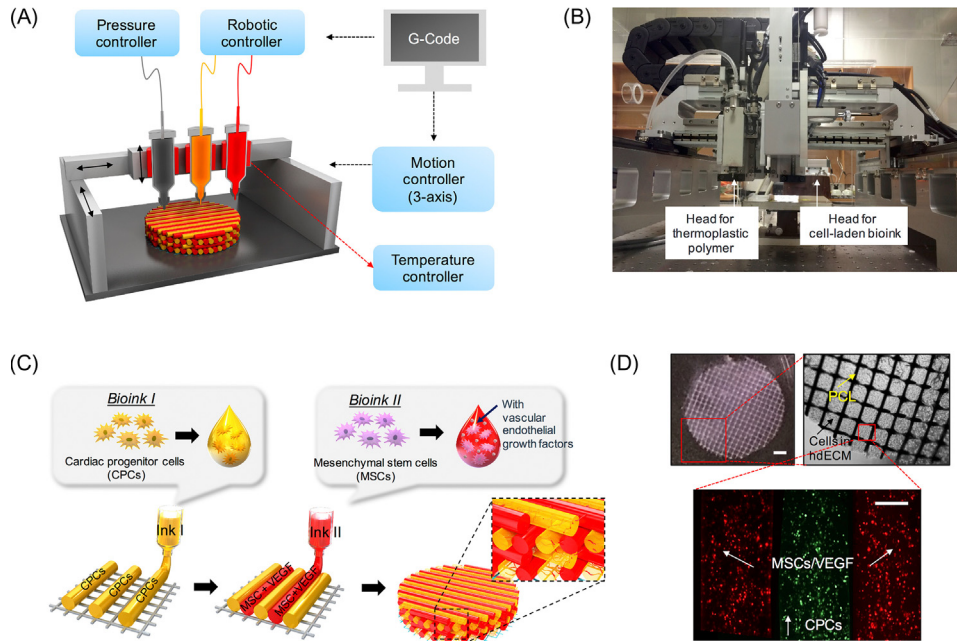


FIGURE 10.3 Schematic of microextrusion bioprinting to create prevascularized stem-cell patches. (A) Microextrusion 3D cell printing system, and (B) macroscopic view of the printer. (C) Illustration of prevascularized stem-cell patch including multiple cell-laden bioinks and supporting PCL polymer. (D) Fabricated patch including the two types of cell-laden bioink and PCL supporting layer [scale bar (left top), 1 mm; scale bar (bottom), 200 μm]. PCL, Polycaprolactone. Source: Reprinted from Jang J, Park H-J, Kim S-W, Kim H, Park JY, Na SJ, et al. 3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair. *Biomaterials* 2017;112:264–74 [70] with permission from Elsevier.

tissue patches are ECM derived [61], but other biomaterials, culture condition, stimuli and arrangements have been used to develop cardiac patches [61].

10.2.2.1 Decellularized materials

Decellularization is a commonly used tool for creating protein scaffolds [73]. Cells can be reseeded onto these scaffolds to create a tissue with a native ECM [73]. Decellularized structures can be used as functional scaffolds for cardiac tissue patches and have been shown to promote tissue repair [74]. Specifically, the decellularized ECM of the small intestine submucosa and bladder matrix have been used to create cardiac tissue patches [23,75–77]. These studies reveal the benefits of using a host's native ECM which include the promotion of new vasculature and cell infiltration post implantation [75–77]. In a study by Ott et al., [78] bioartificial hearts were developed by decellularizing cadaveric rat hearts with a detergent and reseeding the construct with cardiac and EC. This natural scaffold was shown to contract and was characterized in vitro for a month but not further assessed in vivo [78]. Decellularized tissues used as scaffolds for cardiac tissue patches have shown promising results, but the limitations in reseeding and the lack of vasculature are barriers for their application.

10.2.2.2 Electrically conductive materials

Scaffolded tissues have some disadvantages including their lack of conductivity and elasticity. To improve the electromechanical properties, recent studies have incorporated a variety of electrically conductive materials and cells within scaffolds. This includes a chitosan/carbon model [43,79], a nanowire 3D model [80], use of stem cells and higher concentrations of cardiomyocytes [81–84], and scaffold-free models incorporating mainly cardiac and native connective tissues [85]. In one study [60], a patch with micro-needles was produced by molding poly(vinyl alcohol), a biocompatible polymer, to establish a channel of communication with the myocardium. The patch was seeded with cardiac stromal cells (CSCs) and presented as a painless alternative to facilitate the penetration of regenerative factors secreted from the CSCs into the heart [60]. Patch tests were performed in a rat MI model, and the microneedles did not damage heart tissue or incite a significant inflammatory response [60]. Additionally, the application of the patch increased the heart's wall thickness and was suggested to promote cardiac repair [60]. Scaffolding of cardiac tissue patches may be enhanced by incorporating electrically conductive biomaterials.

10.2.3 Construct performance in vivo

This section will provide an overview of the in vivo performance of biomaterials and constructs applied to cardiac tissue. A few essential performance criteria to assess cardiac tissue constructs include the degradation rate, the immunogenicity, the angiogenic, and the arrhythmogenic properties [11,86–88]. Evaluating these criteria will assist in determining the efficacy and the potential for therapeutic use of cardiac tissue constructs.

10.2.3.1 Material degradation

A construct's degradation rate in vivo is essential to its success as a therapeutic treatment. Cardiac biomaterials should be able to withstand the constant perturbations of the heart's surface until the damaged tissue is repaired [11,86,89]. This suggests that there is an optimal window for material degradation [38]. Typically, ECM-derived biomaterials have a shorter degradation period [26]. For example, biomaterials such as gelatin and alginate have significantly shorter degradation periods than chemically modified materials such as MeHA [26]. This can be observed in the study of an injectable biotin-labeled alginate hydrogel [54]. Degradation assays found significantly decreased areas of biotin positive staining at 1, 4, and 6 weeks post injection [54]. In contrast, the hydrogel MeHA was tested in vitro and revealed a loss of fewer than 25% of its mass in a 20-week period [26]. Overall, there is a wide assortment of biomaterials available for use in cardiac tissue engineering with highly variable degradation rates.

10.2.3.2 Immunogenicity: macrophage infiltration

Biomaterial rejection associated with macrophage infiltration is a serious concern for cardiac tissue implants. The immune system, which normally targets foreign body invaders for destruction, has been shown to become activated against various biomaterials [90–92]. In general, immune rejection will occur through neutrophil or macrophage

activation followed by the cellular immune response including inflammation and damage to the foreign material [91]. This reaction however is much more complex and has many interplaying components [91]. Additionally, synthetic biomaterials can trigger T-cell activation without binding as an antigen, perhaps by acting as mitogens [93]. One strategy to reduce concerns of immunogenicity is to use cells and scaffolds derived from the model organism that is being tested in vivo [93]. The ECM secreted by cells is a known regulator of immune responses [93]. The ECM components collagen, elastin, and fibrin act by binding macrophages and monocytes and facilitate the exchange of signaling molecules between cells [93]. Thus, by avoiding synthetic materials and utilizing natural ECM components in a cardiac construct, the immunogenic risk may be reduced.

10.2.3.3 Neovasculature and angiogenesis

The formation of new blood vessels is essential in repairing damaged tissue often seen in MIs or for integration of tissue patches [87,94,95]. Various material designs, biopolymers, and proteins have been used to facilitate this process. [87,94–97]. One of the benefits of incorporating decellularized ECM proteins into hydrogels or scaffolds for cardiac tissue is the observed neotissue formation and angiogenesis [74–77]. A diverse range of engineering methods have been applied to navigate the issue of angiogenesis. Some of these include scaffolds with porous or channeled designs and polymeric materials used to deliver angiogenic protein growth factors in cardiac tissue [87,95]. Overall, despite the various attempts to induce vascularization, angiogenesis remains one of the largest challenges in cardiac tissue engineering.

10.2.4 Scarring and arrhythmogenesis

A major concern of using live cells and biomaterials is arrhythmogenesis [98–100]. Previous studies have shown that scars are a niche for electrical instability and lead to the development of arrhythmia [101–103]. The assessment of arrhythmia is thus essential in reviewing the in vivo biocompatibility of biomaterials for cardiovascular tissue engineering.

10.2.4.1 Assessment of arrhythmogenicity

Arrhythmogenesis in vivo is commonly tested by using burst pacing and extra stimulus pacing [53]. Some researchers [53] have assessed the potential for arrhythmogenesis using programmed electrical stimulation in the left ventricle. These researchers also looked at the return to intrinsic rhythm after nonsustained ventricular tachycardia (VT) and/or the presence of sustained VT [53]. Statistical tests were used to compare the incidence of VT between animals which received the biomaterial and controls to determine if a specific biomaterial is more arrhythmogenic [53]. Given the association of scars with arrhythmias, some authors [88,104] use scar formation or infarct volume as a surrogate marker for arrhythmogenicity. This assessment often has a dual purpose to also evaluate the extent of cardiac repair [104]. In other instances, electrophysiological optical mapping has been used on perfused hearts to directly assess the electrophysiological effects of

biomaterials post injection [105]. These tools present a variety of methods to assess the implications of biomaterials on cardiac rhythms.

10.2.4.2 Arrhythmogenicity of biomaterials

Most live cells are delivered in the presence of biomaterials. In a study evaluating the effect of biomaterials on cardiac rhythms, Rane demonstrated that the injection of a polyester hydrogel led to activation delay, greater heterogeneity in action potential durations, increased dispersion in repolarization, and thus potentially reentrant arrhythmias [105]. In a number of studies [106,107], it is unclear whether the arrhythmia observed is due to the inherent danger of implanting live cells or the implantation of the biomaterial itself [99,100]. Furthermore, this may be complicated by the use of immature stem cells or stem-cell derived cells, where improper or insufficient coupling as a result of immaturity can increase the likelihood of arrhythmogenicity [108]. Apart from the type of biomaterial, some authors have discussed that the location and distribution of biomaterials can determine arrhythmogenicity [109]. Thus, several factors can contribute to the arrhythmogenicity of biomaterials, which should be determined to optimize the properties of the material.

10.2.4.3 Mitigating the risk of arrhythmogenesis

To reduce the risk of arrhythmogenesis, some authors have used novel methods to deliver antisense oligonucleotides to therapeutically relieve ischemic arrhythmogenesis [110]. The use of antifibrotic therapy to reduce scar formation, which is associated with arrhythmogenesis, is another way to reduce arrhythmias indirectly [111]. Despite the potential to cause arrhythmias, the use of biomaterials such as placenta-derived hydrogel has been shown to reduce scar formation and thus arrhythmogenesis [88]. In addition, the injection of biomaterials may preserve the infarct wall thickness [35] and act as a scaffold for implantation of myocardial cells to promote cardiac repair [104,112]. Thus, the ideal biomaterial will promote cardiac repair and reduce scar formation.

10.2.5 Challenges of biomaterials used in cardiac tissue engineering

Challenges that remain for the use of cardiac tissue constructs in vivo include the invasive delivery method, adverse immune response, rudimentary vascularization, limits with engraftment, and electrical coupling [60,61,65,68]. In many instances, despite the contractility, the cardiomyocytes are not mature enough to display the characteristic myofibril regions [71]. Another key issue often seen with tissue patches is the lack of perfusion within the tissues. This is a result of the absence of vasculature present in these constructs. Without the proper circulation system, cells within tissue patches cannot receive nutrients and will often lead to patch necrosis [113]. To navigate this issue, biomaterials have been used to make porous or channeled scaffolds to provide vascularization potential [11,114–116]. Still, interest in patches and biomaterial injections remain and has led to additional proposals such to develop drug eluting patches and alternative, noninvasive, delivery methods [60,65]. Overall, these studies demonstrate the efforts and challenges involved to develop cardiac constructs and highlight some in vivo models used to assess cardiac repair.

10.3 Constructs and biomaterials used in vascular tissue engineering

A quest in the field of vascular tissue engineering has been the development of vessels capable of replacing diseased vasculature. With the advent of biomaterials, tremendous leaps have been made towards reaching this goal [117,118]. The need for vascular grafts as implants arises from the ubiquitous nature of diseased and injured blood vessels [119]. The most common vascular diseases include aneurysms, atherosclerosis, and blood clots including deep vein thrombosis, pulmonary embolisms, coronary artery disease, and stroke [120]. Numerous elements have been associated as risk factors for vascular diseases, some of which include reactive oxygen species, diabetes, hyperlipidemia, and smoking [121–124]. Modern clinical therapeutics used to address diseased vasculature consist of reconstructive operations such as open vascular reconstruction and newly emerging minimally invasive techniques such as stenting, sclerotherapy, and endovenous laser treatment [125,126]. Engineered substitutes have been developed due to the limited growth potential and vulnerable structural integrity of injured vasculature [127]. To find an alternative to these surgical procedures, research within vascular tissue engineering has become focused on designing grafts and constructs capable of replacing diseased blood vessels in vivo [127]. These materials and constructs range from entirely synthetic grafts to scaffold-free cellular vessels [128–130]. Furthermore, a variety of techniques are used to create these constructs including 3D bioprinting, electrospinning, polysurgery, and other methods [131–135]. This section will briefly discuss the various construct designs and biomaterials in vascular tissue that have been tested in vivo.

10.3.1 Biomaterials used in vascular tissue engineering

Recent graft designs have incorporated degradable, nondegradable, synthetic, and organic-based polymers [117,129]. Biopolymers or polymers created from natural materials have become increasingly popular within the last decade due to an increase in various cross-linking methods and their favorable biocompatibility outcomes (Table 10.1) [136]. Some of the most common biopolymers used in vascular tissue engineering include type I collagen, elastin, fibrin, and various other ECM proteins (Table 10.1) [117,130,137–141]. Common synthetic materials used to develop synthetic vascular grafts include expanded polytetrafluoroethylene (PTEE), microporous polyurethane, and Dacron meshes (Table 10.2) [117,137]. Biodegradable synthetic polymers are modified so that they degrade in a temporally predictable manner to allow for tissue growth [157]. The most commonly used biodegradable synthetic polymers include polyglycolide, poly(ether urethane urea) (Lycra), polyethylene glycol, polyglycolic acid (PGA), polylactic acid (PLA), polylactide-coglycolide (PLGA), poly-L-lactic acid (PLLA), and polycaprolactone (PCL) (Table 10.2) [117,137]. Each of these materials have distinct physical properties ranging in elasticity, mechanical strength, and degradation rates which should be considered when designing a vascular graft [117,137,157] (Table 10.2 and 10.3).

10.3.2 Fabrication methods

Various techniques have been used to create vessel constructs, but the most successful include 3D bioprinting (Fig. 10.3) and electrospinning (Fig. 10.4). Electrospinning is a

TABLE 10.2 Selected properties of synthetic, biodegradable polymers investigated as scaffold materials for both cardiac and vascular tissue engineering.

Polymers	Melting point, T_m (°C)	Glass transition point, T_g (°C)	Degradation kinetics	Reference
1. Bulk degradable polymers				
PDLLA	Amorphous	55–60	Sutures are absorbed completely in vivo in 12–16 months	[142–144]
PLLA	173–178	60–65	Sutures can be absorbed completely in vivo after months	[142,144]
PGA	225–230	35–40	Sutures are absorbed completely in vivo in 6–12 months	[142,144,145]
PLGA	Amorphous	45–55	Sutures are absorbed completely in vivo in 2–12 months	[146]
PPF	<140	–20	Not available	[146,147]
PCL	58	70–724	Not available	[148–150]
PHB	177	4	Much slower than PLA, PGA, and PLGA	[151,152]
2. Surface erodible polymers				
Poly(anhydrides)	150–200		Surface erosion	[146,147,153]
Poly(ortho-esters)	30–100		Surface erosion	[146,154]
Polyphosphazene	–66 to 50	242	Surface erosion	[155,156]

PCL, Polycaprolactone; *PDLLA*, poly-D,L-lactic acid; *PGA*, polyglycolic acid; *PHB*, polyhydroxybutyrate; *PLA*, polylactic acid; *PLGA*, polylactide-coglycolide; *PLLA*, poly-L-lactic acid; *PPF*, polypropylene fumarate.

Adapted from Chen Q-Z, Harding SE, Ali NN, Lyon AR, Boccaccini AR. *Biomaterials in cardiac tissue engineering: ten years of research survey*. Mater Sci Eng: R: Rep. 2008;59(1–6):1–37, [6] with permission from Elsevier.

method where a jet of charged polymer solution is created by an electric field and manipulated to fall onto a collecting screen [159]. The fiber diameter may be tailored during the electrospinning process and can range from 0.5 to 5 μm [159]. Thus, electrospinning has been used to create scaffolds and grafts composed of various materials including collagen, fibrin, and the previously mentioned synthetic materials [130,138,139,159]. The most commonly used 3D-bioprinting method for creating vasculature is microextrusion where a hydrogel material is additively extruded through a syringe [160,161]. Using this method, various cell-laden hydrogels composed of alginate, matrigel, or collagen have been used to create vessels [162]. Each of the methods and materials discussed, whether synthetic, organic, electrospun, or cell sheet engineered, have distinct biocompatibility advantages and disadvantages that will be discussed in the following segments.

10.3.3 Construct performance in vivo

Currently, interest is growing to apply vascular biomaterials within a clinical setting. To facilitate the growing demand, this section will discuss the biocompatibility requirements of these materials within the framework of the FDA “Guidance Document for Vascular

TABLE 10.3 Advantages and disadvantages of polymeric biomaterials for tissue engineering.

Biomaterial	Advantages	Disadvantages
Naturally occurring polymers	1. Excellent biocompatibility (nor foreign body reactions) 2. Biodegradable (with a wide range of degradation rates) 3. Bioresorbable	1. Poor processability 2. Poor mechanical properties 3. Immunogenic problem (i.e. poor immune compatibility) 4. Disease transfection
Bulk biodegradable synthetic polymers Poly(lactic acid) Poly(glycolic acid) Poly(lactic-co-glycolic acid) Poly(propylene fumarate)	4. Good biocompatibility 5. Biodegradable (with a wide range of degradation rates) 6. Bioresorbable 7. Off-the-shelf availability 8. Good processability 9. Good ductility	1. Inflammatory caused by acid degradation products 2. Accelerated degradation rates cause collapse of scaffolds
Surface bioerodible synthetic polymers Poly(ortho esters) Poly(anhydrides) Poly(phosphazene)	1. Good biocompatibility 2. Retention of mechanical integrity over the degradative lifetime of the device 3. Significantly enhanced tissue ingrowth into the porous scaffolds, owing to the increment in pore size 4. Good processability 5. Off-the-shelf availability	1. They cannot be completely replaced by new tissue 2. Concern associated with the long-term effect
Nondegradable synthetic polymers	1. No foreign body reactions 2. Tailorable mechanical properties 3. Good processability 4. Off-the-shelf availability	1. Second surgery is required, or 2. Concern associated with the long-term effect if they have to stay in the host organ for a lifetime

Adapted from Chen Q-Z, Harding SE, Ali NN, Lyon AR, Boccacini AR. Biomaterials in cardiac tissue engineering: ten years of research survey. *Mater Sci Eng: R: Rep.* 2008;59(1–6):1–37, [6] with permission from Elsevier.

Prostheses 510(k) Submissions” [163]. In this guidance, the FDA has identified nine criteria to assess the risk of vascular prostheses which include: (1) thrombosis, embolic events, occlusion, stenosis; (2) leakage by hematoma, hemorrhage, and blood leakage; (3) biocompatibility and allergic reaction; (4) graft disruption; (5) seroma; (6) false aneurysm/pseudoaneurysm; (7) true aneurysm/dilatation; (8). infection/sterility; (9) performance. These criteria fall under three main categories including blood clot formation, immunogenicity, and vessel integrity. We use these risk factors as a blueprint for highlighting the performance of vascular grafts in vivo.

10.3.3.1 Blood clots

The formation of blot clots presents a threat to the efficacy of engineered vessels and the survival of the host. Thrombogenic events stop the flow of blood to tissues and result in oxygen starvation ultimately leading to tissue death [164]. Vascular bypass graft failures

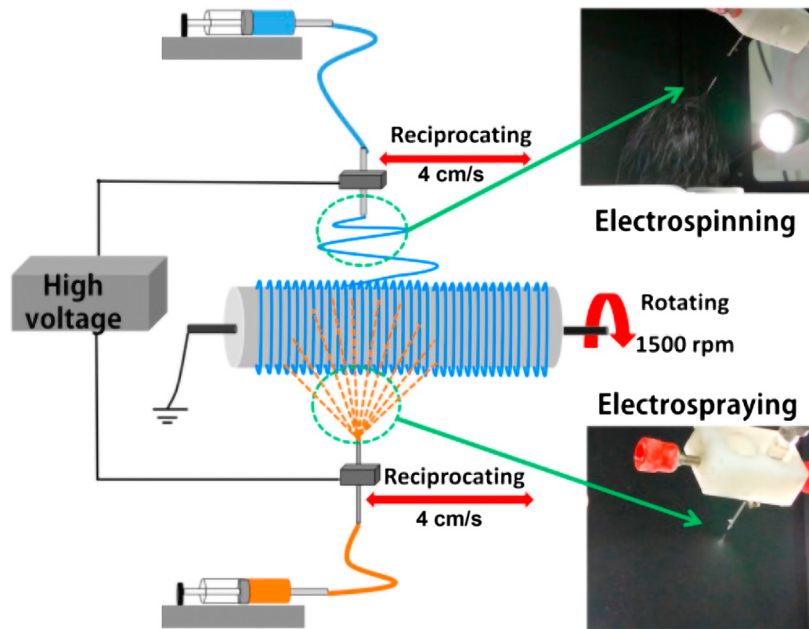


FIGURE 10.4 Schematic of a simultaneous electrospinning and electrospaying experimental setup. Source: Reprinted from Su C, Li Y, Dai Y, Gao F, Tang K, Cao H. Fabrication of three-dimensional superhydrophobic membranes with high porosity via simultaneous electrospaying and electrospinning. *Mater Lett* 2016;170:67–71 [158] with permission from Elsevier.

are commonly a result of blood clot formation, known as thrombosis, under 30 days [165–167] and chronic hyperplasia, or excessive cell proliferation, greater than 30 days following operation [138,168–171]. Therefore, an ideal vascular graft should have antithrombogenic properties, meaning that the graft should prevent aggregation of blood proteins [172]. To mitigate this issue, natural and synthetic materials have been used to coat vessels to create a hydrophilic antithrombogenic microenvironment around the lumen including heparin, fibronectin, and various ECM proteins [166,173]. For example, one study incorporated a hydrophilic polymer layer of polyethylene oxide (PEO) with the globular protein albumin to decrease platelet adhesion to implanted vessels [173]. Grafts can also be made from mixtures of synthetic and natural materials to achieve a highly tunable set of characteristics [174]. In one instance, a PCL/chitosan hybrid small diameter vascular graft was infused with heparin so that the slow release of the molecule mediated antithrombogenic properties [174]. Another method commonly tested is the seeding of cells native to vasculature onto grafts to achieve similar antithrombogenic properties [175]. One vascular graft, electrospun with PCL/collagen, was seeded with EC followed by SMC to mimic the native vasculature [175]. To test the thrombogenic properties of the cell-seeded grafts, the constructs were exposed to arterial flow for 15 minutes followed by characterization using hematoxylin and eosin staining as well as SEM [175]. In the final comparison, bare grafts appeared to have significant platelet adherence compared to those lined with ECs [175].

10.3.3.2 Vessel integrity and aneurysm formation

Disruption or leakage of an implanted vascular bypass graft may lead to hemorrhaging or seroma formation which are associated with very poor survival outcomes [176]. Studies have compared the in vivo performances of various engineered graft models [177,178]. One study compared the disruption of autogenous and PTFE vein grafts for vascular reconstruction [177]. The study found that PTFE grafts had a smaller incidence of anastomotic disruption and were as effective as the autogenous veins [177]. In another study, no graft leakage was observed in electrospun vessels made from PCL/collagen following in vivo implementation in rabbits [175]. Further, scaffold structural integrity and aneurysm formation were evaluated in 2-week intervals following surgery using duplex ultrasonography to measure graft diameter and observe blood flow dynamics [175]. At one month, a CT scan was completed and assured that the vessel maintained a stable 3D structure [175]. This study highlights how PCL/collagen electrospun scaffolds may be seeded with EC in vitro and subsequently tested with in vivo like conditions [175]. These vessels were observed to withstand hemodynamic pressures while maintaining structural integrity [175]. In addition to electrospinning, various strategies have also been adopted for preventing disruption of synthetic grafts. One such method includes a weaving method for creating Dacron aortic grafts [179]. Concerns should also be given to immunogenicity, which has been shown to disrupt grafts [178]. A study observing the tendency of various microbial species to colonize autogenous and prosthetic grafts found that a particular strain, *Pseudomonas aeruginosa*, led to the disruption of 60% of PEO and 100% of autogenous grafts [178]. This study highlights a critical connection between graft integrity and the immune rejection of grafts [178]. Overall, producing a structurally sound engineered vessel involves consideration of the material degradation, fabrication method, and immunogenicity of the material.

10.3.3.3 Immunogenicity

Infections due to graft implants in peripheral surgery may lead to the death or loss of an extremity [180]. A review analyzing 164 cases of infected vascular prosthetics revealed an overall mortality rate of 33.9% or 52 of 153 patients [181]. Those with an aortobifemoral infection had a 23% amputation rate and femoropopliteal infections resulted in a 36% rate [181]. *Staphylococcus aureus* was the most commonly cultured organism [181]. This study highlights why sterility is essential in modern surgical practice, and any materials or cells incorporated in vivo should be treated accordingly [181]. The conventional method of ensuring sterility of biomaterials is autoclaving and has been done with various polymers and hydrogels [182–184]. Biocompatibility requirements for vascular grafts generally include sufficient structural integrity, elasticity, antithrombogenicity, and nonimmunogenicity [185]. Research has warned that microbes such as staphylococci attach to hydrophobic synthetic polymers with strong affinity, and that small infections may flourish when incorporating these materials [186]. Further, a study comparing autogenous and synthetic graft infection outcomes revealed that the type of graft material had a smaller effect on infection outcomes than the specific microbes [178]. This study tested both gram negative and positive microbes in an in vivo canine model where implanted vessels were inoculated with 10^8 colony forming units (cfu) of various

microbe strains and included a saline injection control [178]. The immune system may become activated due to contamination of a vascular graft as well as chance binding against synthetic materials and immune-toxic factors [187]. This is partially why many naturally derived ECM proteins such as collagen, elastin, fibrin, and others are incorporated into vascular graft materials [188]. One in vivo study on the immunogenicity of electrospun grafts from bio-derived materials looked at small diameter silk fibroin vascular grafts implanted in rats [185]. The immune response was measured using inflammatory macrophage and T-cell staining with anti-ED1 and anti-CD4 antibodies, respectively [185]. Data revealed few inflammatory macrophages and an absence of T-lymphocytes within the scaffold suggesting no immune response was activated against the graft [185]. Overall, the immunogenicity of a vascular graft is related to the material, either synthetic or bioderived, and the species-specific origin of the graft proteins.

10.4 In vivo applications of constructs and biomaterials

In vivo biocompatibility tests are used to assess the feasibility of a material for clinical or therapeutic application. In either cardiac or vascular implementation, in vivo assays should be designed to adequately inform researchers of the biocompatibility properties, faults, and complications. Various small and large animal models have been used for in vivo experimentation of cardiovascular biomaterial biocompatibility. The most commonly used include murine, rodent, rabbit, canine, porcine, and sheep models [189–191]. Each model has advantages and disadvantages to consider including the ease of obtaining the desired number of animals, the likelihood of approval by an institutions IRB, the cost, the surgical procedures, and an animal's specific immune requirements [191]. Although swine and canine cardiovascular systems share greater similarities with humans than smaller animal models, the housing costs of large animals are significantly greater when compared to small animal models [191]. Thus, a desired statistical power may be easier to achieve with smaller animal models such as murine or rodents compared to canine or porcine [191,193]. However, the surgical difficulty typically increases as an animal size decreases [191,193].

10.5 Conclusion

Rapid growth in the fields of material science and cardiovascular tissue engineering have led to an increase in potential therapeutics for cardiovascular diseases. Biomaterial applications have shown promising results as various treatments for necrotic heart tissue, scarring, left ventricular remodeling, and other symptoms of MIs. This review has identified a few key properties of successful cardiac tissue biomaterials including adherence, flexibility, integrity, and porosity. Additionally, these biomaterials can improve host outcomes by reducing scarring, preventing arrhythmogenesis, and promoting angiogenesis. The use of biomaterial injections and tissue patches shows promise for the alleviation of MI pathologies. However, perfusion through these constructs in vivo is a challenge, and future studies will likely focus on generating novel methods for angiogenesis. Vascular grafts have been successfully generated using biomaterials to replace diseased vasculature.

The biocompatibility of biomaterials used in vascular tissue engineering have been highlighted with respect to FDA guidelines. With rapid advancements in cardiovascular tissue engineering, the application of biomaterials as a treatment for various diseases will likely become more prevalent.

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Clinical and surgical aspects of medical materials' biocompatibility

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Abbreviations

BDS	biodegradable stent
BMS	bare metal stent
BSE	bovine spongiform encephalopathy
CAD	coronary artery disease
CoCr	cobalt–chromium
CVD	cardiovascular disease
DES	drug eluting stent
e-PTFE	expanded polytetrafluoroethylene
ECM	extracellular matrix
FDA	Food and Drug Administration
HA	hydroxyapatite
ISO	International Standards Organization
LVAD	left ventricular assist device
MRI	magnetic resonance imaging
PERV	porcine endogenous retrovirus
PET	polyethylene terephthalate

PLA	poly-L-lactic acid
PMMA	polymethylmethacrylate
PTFE	polytetrafluoroethylene

11.1 Introduction

The Latin adage “Primum Nihil Nocere”, which is a part of the Hippocratic Oath, was defined by Hippocrates centuries ago and refers to the meaning that first of all, do not harm. It is accepted as the main principle in medical applications [1]. The history of medical devices and biomaterials dates back more than 2000 years albeit significant progress has been made in the last decades. Subsequently, life expectancy of human beings and quality of life have increased, thanks to the improvements in science, medicine and technology [2].

A biomaterial is defined as a substance other than a drug designed as a whole or part of a complex system, which interacts with the living organisms, and functions for therapeutic or diagnostic purposes. In other words, they are materials used in the body with the aim of replacing and/or improving the function of damaged tissues or organs [3,4]. Biomaterials are widely used in clinics and surgery including orthopedic and dental applications, cardiovascular diseases (CVDs), ocular diseases, wound healing, urological diseases and general and reconstructive surgery procedures. They can be either natural or synthetic in origin. Natural biomaterials mostly include derivatives of collagen, fibrinogen, hyaluronic acid, glycosaminoglycans, hydroxyapatite (HA), cellulose, chitosan, and silk fibroin. Polymers, ceramics, metals, and composites are the investigated classes of synthetic materials [3–7]. Briefly, natural biomaterials are composed of extracellular matrix (ECM) components and present low immunogenicity if appropriately decellularized. They usually have better biocompatibility and bioactivity compared to synthetic biomaterials. However, supply of the tissue depends on the availability of donors and takes time to prepare. In addition, there is a more evident risk for microbial contamination during tissue preparation. On the other hand, synthetic materials can be used as “off-the-shelf” products with less contamination risk. Besides, there is a chance to control over material’s biomechanical features. However, microarchitecture and microvasculature usually do not resemble the native tissue, and there is an increased risk of foreign body reaction with synthetic materials [7]. In addition to be sorted according to their origin and chemical structure, they can also have different characteristics, which allow different classifications such as resorbable, bioinert, bioactive, biostable, biodegradable, and sterilizable materials.

Regardless of their properties, biomaterials must be biofunctional, which make them suitable for application to carry out a specific function in the organism, either as an implant or a device at the required time period. For example, an implant designed for cosmetic surgery application must have space filling properties, whereas a cardiac pacemaker must have electrical stimuli and conductive properties to fulfill its design requirements. Besides, biomaterials must integrate with the organ or tissue allowing for long-term use without the rejection by host either by allergenic/inflammatory reactions or losing its functionality [3,4].

Biocompatibility, which defines the ability of a material to perform its function in a specific application with an acceptable host response, has gained special attention in recent years in the era of material science [2,5,8–10]. It is one of the exigencies for a material to perform its specific function and reflects the grade of interaction between the applied

material and host response. Therefore it is one of the corner stones in biomaterials research [5,9–11]. In fact, our perspective to biocompatibility term has evolved over the years. At first, a completely inert material to the organism, which does not evoke any response in its biological environment, was considered to be biocompatible. However, during the following years, it was recognized that any material in the body regardless of its function or characteristic always evokes a kind of response, which varies according to application type or patient characteristics such as age, sex, or comorbidities [12,13]. Therefore, biocompatibility refers to the interaction between biomaterials and living systems, related with an acceptable harm to the organism. In other words, biocompatibility reflects a set of characteristics to use a material safely in a living organism [3,13].

Biomaterial-host interaction starts with the application of the material in a way such as surgical implantation, infusion or injection of the biomaterial into the organism. This process initiates a response in the host tissue, which may be due to mechanical forces, protein adsorption, cell adhesion, or degradation of the material. Subsequently, the host response progresses with variable kinetics such as inflammation, hyperplasia, thrombosis, and/or calcification of the material. If this response is resolved with clinically acceptable effects, biomaterial is considered to be tolerable to patient. If resolution of the host response is inadequate with clinically unacceptable outcomes, the material is considered to be nontolerable. Basically, these processes constitute the biocompatibility pathway of biomaterials [9,14]. Cellular mechanisms of this pathway are closely linked with the components of the biomaterial, which interacts with the surrounding tissue. Cell internalization mechanisms such as phagocytosis, endocytosis, pinocytosis, or direct transit through the plasma membrane transmit the component into the cell. The mechanism of this internalization process may vary according to the type and interaction of materials and cells. Nevertheless, it is controlled by physical and chemical parameters such as size, structure, volume, and/or shape of the material. Although it is possible that the material component surrounded by vesicles may be removed from the cell by the activation of lysosomes and endosomes, it may also induce various pathological alterations in the cell. Reactive oxygen species' formation and subsequent cell damage, alteration in organelle functions, activation of apoptotic and necrotic processes, passage into nucleus and subsequent gene damage with alteration in gene expression profiling are the main pathophysiological mechanisms during this course [9,15,16]. In addition to these biochemically-mediated cellular alterations, it is possible that there may be mechanically induced effects of the material component and cell interaction, which may be guided through the ECM molecules. However, it should be noted that this mechanotransduction is finally converted to chemical signals by cell and nucleus, which cause similar biochemical and cellular alterations as described above [9,17].

Biocompatibility evaluation of biomaterials includes quantification of the extent and severity of the adverse changes in the homeostatic mechanisms that establish the host response. In other words, biocompatibility tests are performed to determine whether the medical device/material performs the function regularly and does not harm the patient. Briefly, there are three major biological responses that need to be considered for biocompatibility assessment: inflammation, wound healing, and immunological reactions and/or immunity. A biomaterial or medical device implanted in the body must achieve some significant goals to be defined as biocompatible and appropriately functional in the host environment. It must restore the target tissue while performing appropriate function. Besides,

it must inhibit the foreign body response caused by macrophages and/or foreign body giant cells, inhibit scar and fibrous capsule formation, and immune system response, which may affect the functionality of the device [8]. Biocompatibility assessment requires various methods including complex *in vitro* and *in vivo* experiments, which are standardized by various organizations such as American Society for Testing and Materials, British Standards Institute, International Standards Organization (ISO), and US Food and Drug Administration (FDA) Agency [10,18]. These analyses involving *in vitro* and *in vivo* experiments mainly aim to test cytotoxicity, sensitization, irritation, systemic acute or chronic toxicity, genotoxicity, hemocompatibility, carcinogenesis, reproductive and developmental toxicity, and biodegradation [10]. Basic aspects of biocompatibility with evaluation and test methods of biomaterials are briefly summarized in Fig. 11.1. Understanding the biocompatibility issues of biomaterials is an important task for the clinicians and surgeons, who actively use these devices to treat their patients. Therefore, this chapter aims to provide information and guide the surgeons and clinicians about biocompatibility issues of specific biomaterials and medical devices widely used in the clinics and operation rooms.

11.2 Orthopedic biomaterials

Biomaterials, which are used to repair defects related with skeletal system are considered orthopedic biomaterials [19]. It is possible to repair a significant portion of bone

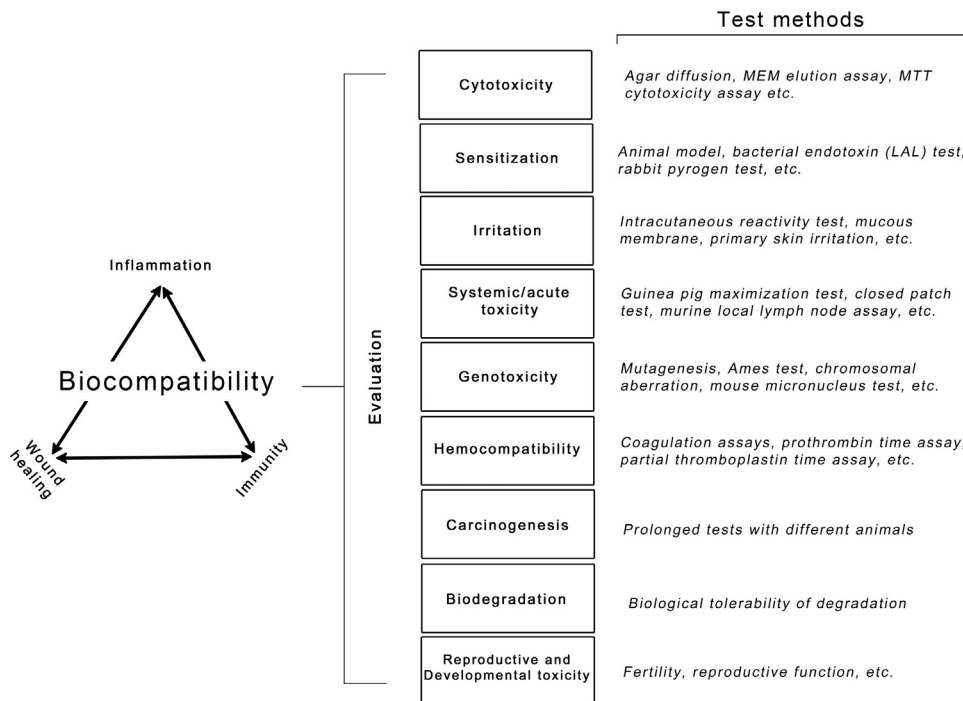


FIGURE 11.1 Basic aspects of biocompatibility with evaluation and test methods.

tissue, joint and/or cartilage-related defects caused by injuries/trauma, developmental anomalies, aging associated changes by using orthopedic biomaterials. Biomaterials developed from metals, ceramics, synthetic/natural polymers, and natural tissues have been widely used in clinical orthopedic procedures for decades. Mechanical features (architectural structure, elasticity, strength, etc.), biodegradation, and surface properties of biomaterials are important points, which determine their applicability. Their physical characteristics can be optimized during the production phase. In addition, they must be biocompatible due to the fact that they will be in constant interaction with the host's biological system after surgical application [20].

The development, definition, and concept of biocompatibility have been discussed in detail. In addition to the host immune response, parameters such as integration of an orthopedic biomaterial with host bone tissue, waste product formation causing periprosthetic osteolysis, and aseptic relaxation are also important biocompatibility issues related with longevity of the material. Orthopedic biomaterials, which are widely used in trauma, arthroplasty and/or spinal applications, can be classified as (1) fracture fixation, (2) joint replacement, (3) graft (auto-, allo-, xeno-), (4) synthetic graft, and cement materials [21].

11.2.1 Fracture fixation applications

Hip, vertebrae, and ankle-related bone fractures are the most common orthopedic problems with a cost of \$10 billion a year just in the United States. It is well known that natural human bones have the capability of regeneration after minor traumas but not fractures including multiple, transverse, and/or large fractures. In such cases, fixation surgery is performed in order to establish the natural anatomy with the help of biomaterials [22,23]. It is possible to provide the stability required for primary bone healing with the use of materials such as staples, intramedullary nails, plates, screws, pins, and cables, which are developed from various raw materials [24,25]. Although a few polymer-based biomaterials are considered for fixation surgery, metal-based orthopedic biomaterials dominate the clinical market, given their ability to mimic the mechanical strength of natural bone [26,27].

Material properties including biocompatibility, biological, and technical characteristics are well defined with the standards of ASTM F2066, ISO 5832-1, ISO 5832-2, and ISO 5832-11 [28]. Stainless steel and titanium biomaterials are the most widely used materials in fraction stabilization surgeries [26,27]. It is known that titanium and titanium alloys are superior to stainless steel in terms of biocompatibility. Infection incidence of stainless steel materials is not at the desired levels [29]. In addition, stainless steel fixation materials contain 13%–16% nickel. This may lead to allergic reaction after application due to the fact that one in five people are sensitive to nickel. Therefore, titanium and its alloys are mostly preferred instead of stainless steel-based materials for fixation surgery and considered more biocompatible. On the other hand, metal compounds also carry biological risks depending on the duration of the interaction in the host system. Due to the fact that stainless steel materials have relatively low corrosion resistance, toxic substances such as nickel, chromium, and cobalt may be released [30,31]. In addition to the limitations of metals used in fixation surgery given their biological characteristics, osteointegration capabilities

of these biomaterials are limited. There are various surface modification strategies to increase the degree of osteointegration. Ceramics such as HA, the leading inorganic component that predominates the natural bone tissue, can be used as surface coating material. These ceramics have the potential to be used alone in various orthopedic applications thanks to their high biocompatibility and ideal biodegradability. However, because of their fragile characteristics, they are not convenient to be used alone in fracture fixation surgery. On the other hand, it is known that coating with ceramics can increase osteointegration capacity and decrease infection risk. However, it should be noted that ceramic-based coating material carries the risk of fracture after surgery [32–34].

Nowadays, metals appear to be the only appropriate option for fracture fixation surgery. However, clinical data associated with their biocompatibility are limited regarding their application area and durability. In addition, all metal-based orthopedic biomaterials carry limitations including magnetic resonance imaging (MRI) or computed tomography related difficulties. Besides, fracture fixing materials must be removed in the long term, which need additional surgery and limit their applicability. In this case, a secondary surgical procedure is one of the limitations that must be considered [35].

11.2.2 Joint replacement applications

Joint replacement surgery is widely performed in the orthopedics clinics if the damage is in the joints. The main goal is to relieve the patient's symptoms as well as to restore the joint function by placing a permanent biomaterial (prosthesis/implant) and improve the quality of life. Similar to fixation surgery, metals are frequently used in joint replacement procedures. Alternatively, natural and synthetic bone grafts from various sources are also applied [23,24]. Total hip replacement is one of the most common surgical procedures for joint replacement operations worldwide. The choice of appropriate biomaterial for total hip replacement may vary according to the clinical and experimental evidence, *in vivo* experience, and the surgeon's expertise [24].

The design of materials used in kinetic/dynamic joints such as knees may differ from hips. Biomaterials, which mimic the normal anatomy of both sides such as the ball-socket system are preferred. On the other hand, biomaterials used in knee replacement may cause unexpected reactions in the host tissue such as eczema and allergic reactions, which are associated with implant failure [36–38]. Regional dermatitis reaction, erythema, urticaria, muscle necrosis, pain, and chronic inflammation may also be observed in patients after application [39–41].

Raw material characteristics of the biomaterials used in joint replacement surgery should be well understood in order to deal with their biocompatibility issues. A significant portion of the biomaterials used in replacement surgery is cobalt–chromium (CoCr)-based metallic alloys also containing molybdenum. Due to the fact that their mechanical properties are better than stainless steel-based materials, they are usually preferred in joint replacement applications. In addition, corrosion resistance of CoCr-based biomaterials is superior when compared to that of stainless steel and titanium. However, their biocompatibility and osseointegration characteristics are still controversial because of their contact time within the physiological system. Therefore, the flexibility and biocompatibility

capacity of CoCr-based biomaterials compared to that of titanium and stainless steel remain problematic [42–44]. Nevertheless, high elasticity and low hardness ratio of pure titanium, which may cause cytotoxic effects, limit their use in joint replacement and this limitation is tried to be overcome by composite biomaterials. For this purpose, vanadium titanium alloys are used in the clinical settings [45].

The cytotoxic and biological effects of metallic material used in surgery are closely related with the degree of metal-on-metal interaction. If the metal-on-metal interaction is at a high level, this may lead up to alterations in the brain tissue structure and neurological problems, especially with CoCr-based biomaterials through an increase in Co and Cr levels in the host blood [46]. Although CoCr-based materials have been widely used in the clinics for more than 50 years, today it is known that they may cause cobalt toxicity after arthroplasties, and the chromium which can be found in various chemical forms in the body, can cause respiratory diseases, renal failure, and hematological defects [47,48]. On the other hand, some studies suggest that this problem can be overcome by implementing coating methods. The application of surface coating of ceramics such as aluminum and zirconium in the metal-on-metal implant is considered to be one of the alternative approaches to overcome toxicity. By this way, it is possible to improve the biocompatibility by preventing mechanical abrasion, reducing toxicity, and subsequently prolonging implant life [49]. In addition to CoCr and titanium, nitinol-based biomaterials are also used in joint replacement applications. Nitinol is a nickel containing material and has good biocompatibility properties, although some clinical reports have revealed adverse effects associated with nickel release [50].

Although most of the biological limitations are associated with the time period, implant material remains in the host, many metallic implants are reported to remain for up to 20 years without causing any complications or infections in the host. This time is mainly related to osseointegration of the material to the natural bone tissue. Although ceramics (such as zirconia, silicon carbide) positively influence biocompatibility and osseointegration, they cannot remain in the structure as whole and separate from the material in due course [34,51]. On the other hand, after optimization of metal-on-metal binary systems and total hip prosthesis with alumina, it was found that the structure maintained its stability without any complication for years [52].

Polymer-based (polyethylene) materials and ceramics are also known to be the initial products approved for clinical usage by the FDA in the 1990s [52]. Although ceramics have significant biocompatibility and integration advantages, there are reports that infections may occur at the border of the bone-marrow after implantation [53]. In this context, it is possible to interpret that the application of an implant usually brings the risk of microbial infection [54]. Therefore, the sterility of the implant surface during the operation or the sterilization of the implant before the application phase is important. In addition, local management of the host immunity prior to surgery could minimize the risk of microbial infection. The main aim should be to regulate the acute/aggressive response in the tissue region, where the biomaterial is applied. Following surgery, a biofilm layer could be formed prior to bone formation, which cannot be eliminated by immune cells, subsequently causing chronic inflammation. It is very difficult to remove this biofilm structure without the use of powerful antibiotics [55]. In order to prevent this phenomenon, various additional materials or antimicrobial surface coatings are applied. In this context, coating

of the material surface with metals such as silver, which is a well-known antibacterial agent and tolerated after ingestion, or other antibacterial agents are recommended as alternatives [56,57].

11.2.3 Graft applications (auto-, allo-, xeno-)

Graft applications using either autografts, allografts, or xenografts comprise the most common tissue transplantation procedures after blood transfusions. Approximately 2 million grafting operations are performed each year [58–60]. Although there are various alternative tissue sources, the structure that best meets the mechanical and biological properties of natural bone is undoubtedly the bone itself [61]. Autogenic bone grafts containing bone tissue collected from different parts of the host, which have osteoconductive, osteoinductive, and osteogenic properties, are widely used. Spongy and/or nonvascularized/noncortical bone tissue are usually preferred for this purpose [62]. Autogenic graft application is still accepted as the gold standard in bone tissue repairs as in other autogenic tissue transplantation procedures. From a biocompatibility perspective, there is no risk for immune response or disease transmission [63,64]. However, autograft applications could have limitations depending on the size and location of bone damage. The need for additional surgical procedure, complication risk, limited available tissue, comorbidity situations are major drawbacks hampering their use. Autogenic bone graft-related complications, including chronic pain, paresthesia, infection, inflammation, arterial injury, ureteral injury, fracture, pelvic instability, cosmetic defects, and hematoma formation have been reported in the literature [65–67].

Allografts are an alternative source for bone grafting and occasionally obtained from cadavers. Osteoconductive allografts are available in a variety of forms or shapes, such as dust, chips, and spongy cubes obtained from cortical and/or bone tissue. However, the pre-processing, which causes the loss of cellular components and bioactive factors contributing to the regeneration process, limits their regeneration capacity [68,69]. Mineralized or demineralized, fresh, fresh-frozen, or freeze-dried allograft options are available in the clinical market. Tissue form and architecture in the frozen or freshly frozen bone tissues are similar to the natural structure. On the other hand, various forms of demineralized bone matrix structures consisting of different mechanical properties are present [70]. Freshly frozen form or dried bone grafts have been reported to assure a high vascularity rate, higher bone integration, and tissue regeneration capacity than fresh allografts [71]. Clinical cases examining biological activity have demonstrated that the application of freeze-dried allogenic grafts is associated with decreased risk of immunological reactions and viral transmission in the donor. Disease transmission risks, such as HIV, Hepatitis B, and Hepatitis C virus infections are still an emerging problem [69,72–74]. It is practically possible to eliminate transmission risk in allogeneic grafts. For this purpose, bone tissue grafts can be treated with organic solvents in the processing stage, as well as in the sterilization stage by ethylene oxide or gamma radiation. Although the risk of disease transmission decreases during these pretreatment stages, the osteoconductive and osteoinductive properties, and subsequently the regenerative capacity are impaired because of the disruption of cells and proteins [75]. Another existing problem is the high cost associated with

the processing course of allogeneic transplants including storage, processing, and sterilization procedures.

One available alternative for orthopedic graft applications is the use of xenogenic grafts, which involve animal-derived tissues. At present, pig, bovine, and horse bones remain as graft sources for bone substitution in clinical applications [76]. From a theoretical perspective, xenogenic biomaterials have advantages in terms of unlimited sources of raw materials, regulation of biocompatibility, and control of disease transmission during the product development phase. On the other hand, inevitably occurrence of animal-related active factors even at a low level increases the risk of immune response in the host. The possibility of viral disease transmission such as porcine endogenous retrovirus (PERV) viremia and bovine spongiform encephalopathy (BSE) are still important issues hampering xenograft use. In addition, ethical concerns are prevalent in the context of patients' beliefs regarding animal source [77,78].

Similar to the allografts, additional procedures can be applied to eliminate the risk of immune response and infection in xenografts. However, these processes alter the osteogenic and osteoinductive properties of the material. Accordingly, the clinical efficacy of xenografts is also limited [63]. Bovine-derived bone grafts are advantageous with an osteoconductive structure based on crystalline HA, but there is an inevitable theoretical risk of BSE, a neurodegenerative disease caused by prions. However, its incidence is at a very low level [79] considering the fact that disease transmission risks can be eliminated through disinfection and sterilization processes during the product development phase. Xenografts for bone applications have been included into the Type 4 group by the World Health Organization, meaning that this group of products does not carry risk for BSE development. Although there is no current clinical report, there could be a risk for PERV and Ebola virus transfection from pig sources, which subsequently could lead to cancer development because of mutagenesis and immunosuppression [80].

11.2.4 Synthetic grafts and filling material applications

Most of the biological grafts and metallic materials do not carry significant risks associated with biocompatibility. However, their biofunctionality could be limited in some cases, leaving adverse effects to be overcome. Thus, there is a general assumption that biofunctionality problems may not be overcome. Synthetic orthopedic biomaterials constitute an important part of the grafting and filling applications. Synthetic orthopedic grafts are mostly developed from calcium phosphate-based ceramics, with well-defined osteoconductive properties [81].

Different than auto and allografts, synthetic grafts can be produced in desired amounts and easily sterilized. The most important advantage of these materials is that they do not have the risk of disease transmission. They can be produced in a variety of biomaterial forms, which can mimic the mineral composition of the natural bone. Ceramic-based biomaterials can be fabricated in different compositions capable of transporting bioactive factors, which can prevent infection development and/or accelerate the regenerative process. There are numerous studies based on the integration of different antibiotics into/onto these materials and their use for the prevention of bacterial infections. In addition,

products for filling bone voids after orthopedic trauma, surgery, or hematogenic osteomyelitis are also available [82].

In addition to calcium phosphate-based ceramics, several other synthetic materials such as calcium sulfate, calcium phosphate-based cements, and bioactive glass have also been used in clinical applications [83]. Calcium sulfates have been used as support material for filling bone defects in orthopedic bone grafting procedures for more than a century. They have a high absorption rate in the host tissue after implantation and exhibit poor mechanical characteristics. Therefore, their use is limited and are preferred only for filling small bone damages or internal bone fixation [84]. Biologically, calcium sulfates are reported to have a high contribution to vascularization and regeneration at the damage site after application. However, increase in blood calcium levels subsequently causing infection is also reported [59,85].

Calcium phosphate-based ceramics can be prepared in different forms such as, porous or nonporous, granular particles, or powder. They are defined according to the calcium–phosphate ratio they contain [86]. HA constitutes approximately 50% of the natural bone and resembles natural spongy bone in terms of mechanical properties. The high calcium–phosphate ratio and crystalline structure in HA-based ceramics delay its absorption after surgery. This delay impairs the mechanical stability after application. Therefore, a HA-based material usually cannot be used alone in orthopedic applications. It can be used as a coating material in order to generate osteoconductive and osteoinductive properties in replacement or fixation materials [87]. Tricalcium phosphate-based bone materials are converted partially to HA in the host, decreasing the level of degradation. They are usually preferred for filling defects in partial dimensions after bone resections [88].

In contrast to synthetic grafting and coating applications, ceramics are used as filler/cement materials after fixation, resection, and grafting. Calcium phosphate-based materials appear to be prominent in the clinics as a filler material. Calcium phosphate-based filler materials are cement-like structures obtained by using a fluid binder [83,89]. As well as being porous and elastic, they are well known to be biocompatible. These materials are osteoconductive and can be injected and molded without the need for any secondary procedure [90]. They are produced in injectable form to fill the bone defect and solidify through an isothermal reaction after application, which make them advantageous in the field of minimally invasive surgery [91]. Unlike grafts, these materials can be used in percutaneous vertebroplasty and kyphoplasty operations [92–94].

There is no significant limitation regarding the biocompatibility of filling materials. However, liquid–solid phase separation may occur during injection in orthopedic applications, which could limit their functionality. Additionally, filling material may separate in the host because of interaction with blood or body fluids after the application [95]. This may cause serious side effects such as inflammation and embolism [96]. Preclinical studies aim to optimize the components in order to overcome such problems. Physical properties of the fluid phase used as carrier or binder can be modulated, besides the size and shape of ceramic particles can also be optimized. Finally, tissue engineering approaches focusing on integration and bioactivity can also be applied [83,97,98].

In addition to filling applications as fixation or replacement support, cement materials could be preferred in a number of applications. In some reported cases, calcium phosphate-based approaches have been used in balloon kyphoplasty operations instead of polymethylmethacrylate (PMMA) cement, which is another filler material. It is also known

that these materials, which integrate well in the spine without any signs of osteolysis or osteonecrosis, may cause demineralization [99]. PMMA has been one of the synthetic materials in common use with low degradation rate in orthopedic surgery from past to present. It is applied as a grout rather than a cement structure for the purpose of filling for orthopedic purposes. Therefore, it can be used as a fixation material in replacement procedures and in vertebroplasty applications [100,101].

11.3 General and reconstructive surgery biomaterials

Biomaterials used in general surgery, as well as in plastic and reconstructive surgery applications are usually derived from human or animal tissues having natural biological properties and the ECM structure, on the other hand some of them are of synthetic origin. An ideal biomaterial used in these applications must be easily transferrable to the host, suitable for remodeling and should induce acceptable levels of immunological or inflammatory response at the host [102]. Biomaterials are mostly used as injectable soft tissue fillers, mesh materials, and breast implants in the general and reconstructive surgery clinics. Biocompatibility issues associated with biomaterials used in general and reconstructive surgeries are briefly summarized in Table 11.1.

11.3.1 Injectable biomaterials

Repair of soft tissues and regaining the former volume are important issues in reconstructive surgery applications. Permanent materials such as silicone and PMMA or temporary materials such as collagen and hyaluronic acid are usually used to sort out the problem [103]. In recent years, the use of injectable soft tissue fillers has become common in the repair of tissue damage caused by trauma, surgery, and aging. The use of injectable soft tissue fillers comes to the forefront because of the possibility of in situ and noninvasive treatment, filling of complex damages, and easy transfer of desired cells or therapeutic agents. Injectable fillers also attract attention because of the long recovery periods and the high complication risks of the invasive methods [104].

Injectable soft tissue fillers used in the operation rooms and clinics should have a number of properties to be defined as an ideal biomaterial. Briefly, they must be biocompatible causing minimal immunological reactions with acceptable toxicity. They must have similar mechanical properties with the target tissue, suitable permeability with pore size and connection, and low cost. Additionally, they should be biodegradable and easy to apply [104].

Injectable biomaterials used in soft tissue healing are classified in four classes as collagen, hyaluronic acid, autologous fat grafts, and synthetic biomaterials according to the source from which they are obtained [104]. Natural injectable biomaterials are mostly derived from collagen, which is abundant in the ECM of skin cells and approved for clinical usage by the FDA [104,105]. Collagen is usually isolated from three sources as bovine, pig, and human. The FDA approved bovine collagen as a tissue filler in 1981. There are various examples of commercially-available injectable biomaterials derived from bovine collagen, used to remove scars, crow's feet, and superficial lines [106]. However, because

TABLE 11.1 Biocompatibility issues related with biomaterials used in general and reconstructive surgery.

Type of biomaterials	Biocompatibility issues	References
Injectable materials	– An ideal injectable material must induce minimal immunological reaction with acceptable toxicity. It must have similar mechanical properties with the target tissue, low viscosity, stable structure, suitable permeability with pore size and connection, be easy to apply and should be biodegradable.	[104] [104, 108–110,113]
	– Hyaluronic acid derived fillers cause low level of immunological reactions but quickly degrade after application	[115] [122]
	– Autologous fat grafts show minimal immunological reactions but are not durable	
	– Polymethylmethacrylate microspheres are phagocytized by macrophages causing inflammation and foreign body reaction, in cases where the size of the microspheres is smaller than 20 μm	
	– Calcium hydroxyapatite derived injectable biomaterials have high biocompatible features due to its similarity to human bone structure	
Breast implants	– Stable implants have been shown to have higher constancy and better preservation compared to saline or fluid implants	[133] [134]
	– Textured breast implants are known to enhance tissue adhesion, promote the proliferation and growth of blood vessels, and increase cell migration and fibroblast adhesion	[136–138]
	– Implant application is associated with problems such as capsular contracture, infection, hematoma, seroma, rupture and migration of the implant	
Mesh materials	– Polypropylene meshes induce chronic inflammatory reactions resulting with foreign body reaction, chronic pain and decreased quality of life	[152,153] [156,157]
	– Biological meshes have better physical and mechanical properties, significantly reduce network-related complication rates, and can be used in contaminated or infected surgical areas. The main limitations are their unpredictable response at the host and high costs.	[163–165]
	– Low-density and large-porous ($> 1 \text{ mm}$) meshes cause less inflammatory response and complications than conventional high-density and small porous meshes	

of their xenogeneic properties they are prone to cause foreign body reaction. There are also commercially-available pig collagen-derived injectable biomaterials, which were demonstrated to have better antiwrinkle potential and fewer side effects compared to bovine collagen-derived biomaterials [107]. Another collagen-derived natural biomaterial is human origin which is effectively used for the treatment of superficial and deep wrinkles. The biggest disadvantage of human collagen-derived biomaterials is their high price [104].

Hyaluronic acid is an alternative source of injectable biomaterials since it causes a low level of immunological reaction [108]. It is widely used by surgeons since its approval by the FDA serving as a bulking agent and tissue former [109]. Hyaluronic acid-derived fillers allow permanent renewal of the tissue at the end of a single application. Therefore, they are classified as temporary soft tissue fillers. One of the major disadvantages of hyaluronic acid-derived fillers is their high rate of degradation after application [110]. In order to reduce the degradation rate and improve the biological performance of hyaluronic acid-derived fillers, the use of crosslinking methods is applied as a solution [111].

In addition, combination with various materials such as gelatin, chitosan, and cellulose can reduce its degradation rate [104]. It is also known that the use of hyaluronic acid with different materials increases the biocompatibility and the volume of the finished product [112]. Hyaluronic acid-derived biomaterial injections may cause tolerable local side effects such as pain, bruising, and edema [104].

Autologous fat grafts are valuable tools in the plastic, reconstructive, and esthetic surgery applications because of their biocompatibility, regenerative potential, and low cost. They are easy to obtain, do not show significant immunological reaction, and used to treat contour disorders, volume loss, breast reconstruction, facial hemiatrophy, lipodystrophy, wound scars, and rejuvenation [104]. The major problem associated with the autologous fat graft usage as an injectable biomaterial is the variable durability of the transferred fat tissue [113]. In order to prolong the durability of the grafts, adipose tissue must be taken in a nontraumatic way, should not be manipulated extensively in order to ensure appropriate vascularization, and its small amounts should be injected into a region close to the muscles, possessing a suitable layer structure [114].

The most prevalent synthetic injectable biomaterials used in plastic and reconstructive surgery applications are composed of PMMA, poly-L-lactic acid (PLA), and HA. PMMA is one of the highly biocompatible and nonbiodegradable polymers. It is occasionally used as an intradermal filler in surgeries [104]. PMMA microspheres are phagocytized by macrophages causing inflammation and foreign body reaction, in cases where the size of the microspheres is smaller than 20 μm . [115]. Therefore, it is reasonable to state that PMMA microsphere size plays an important role in the prevention of phagocytosis and foreign body reaction. The risk of phagocytosis and granuloma formation has been highly reduced in the new generation PMMA-derived tissue fillers making them more biocompatible [116,117].

PLA, which is a biodegradable thermoplastic polymer, has a wide range of applications in plastic reconstructive, orthopedic, neurological, and craniofacial surgeries. Although it has been used as a suture material and screw for many years, its use as a soft tissue filler was started in 2008 by the approval of the FDA [118,119]. PLA tissue fillers are known to increase collagen production and vascularity [117]. They are used to treat nasolabial folds, facial lines and wrinkles, and contour disorders [120]. However, the use of PLA tissue fillers has been associated with dermal fibroplasia after the degradation of PLA microparticles within a few months [119,121].

HA containing injectable biomaterials have high biocompatibility features because of their similarity with the human bone structure [122]. They are mostly used for the treatment and bulking of facial wrinkles [123]. HA microspheres support the formation of new tissue by storing collagen, resolving into calcium and phosphate ions at the injection site within 2–3 months. These ions are phagocytosed by macrophages and are more effective in the treatment of nasolabial folds compared to collagen alone [124,125].

From a biocompatibility perspective, it should be kept in mind that natural injectable biomaterials are advantageous as nonpermanent soft tissue fillers due to their biodegradability, and various biological properties. However, they do not possess structural integrity which is needed for a long-term and persistent effect. On the other hand, synthetic biomaterials do not exhibit strong cell affinity as natural biomaterials. However, there are various synthetic biomaterials designed to incorporate desired mechanical and chemical

properties [104]. Besides, an ideal injectable soft tissue filling material should not trigger a severe inflammatory response and must be easily transferrable to the host tissue [126].

11.3.2 Reconstructive breast surgery and breast implants

Reconstructive breast surgery is one of the most frequently performed procedure in plastic surgery, usually applied for oncological or esthetic purposes [127]. Reconstructive breast surgery can be performed using autologous tissues or breast implants. Reconstruction with breast implants includes one- or two-step procedures. In one-step application the implant is directly placed, and in two-step application the permanent implant is transferred to the patient after the tissue is expanded [128]. One-step application has become a prevalent treatment in most reconstructions [129], while silicone is the prominent material used for this purpose [130]. Over time, five generations of breast implants have been developed, and better results have been obtained in each subsequent generation [131]. Breast implants can be classified according to their filling material (silicone or saline), their shape (anatomical or round), and surface textures (textured or smooth) [132]. Saline implants are formed by combining silicone elastomers and then filled with sterile saline during operation to obtain the desired volume [127]. In silicone implants, silicone can be in fluid or stable form. It does not have sufficient adhesion capability to maintain the natural anatomical shape in liquid form. However, the stable form of silicone implants has higher viscosity, and consists of a greater number of crosslinked silicon monomers [132]. Stable implants have been shown to have higher stability and better preservation compared to fluid (saline)-containing implants [133].

Smooth breast implants are capable of natural movement at the site of implantation. Textured breast implants are known to enhance tissue adhesion, promote the proliferation and growth of blood vessels, and increase cell migration and fibroblast adhesion [134]. The stability of textured implants is thought to be higher than that of smooth implants. This is explained by the fact that the texture process prevents the rotation of the implant and its migration [132].

Implant application in breast surgery is less invasive compared to autologous tissue grafting and the morbidity risk is minimal [135]. However, it has been shown that there may be issues associated with biocompatibility and biofunctionality, such as capsular contracture, infection, hematoma, seroma, rupture, and migration of the implant [136–138]. The most devastating of these complications is the development of capsular contracture, which can cause breast hardening, pain, loss of shape, and volume [128]. Capsular contracture development is observed in 4%–17% of the patients [139]. In addition, the treatment method (radiotherapy, chemotherapy, etc.) applied to breast cancer patients may increase the complication rate because of the selected implant and the risk of complications for each implant may be different [140–142]. Besides, there is no common opinion for which type of implant is most suitable for use.

11.3.3 Hernia repair and mesh materials

Hernia repair operations are widely performed in the general surgery clinics [143]. Tension-free mesh repair is the gold standard for hernia operations and the Lichtenstein

technique is still one of the most preferred treatment modalities. The main advantages of this technique are the low possibility of hernia recurrence and low cost [144]. However, the technique needs an open surgical procedure requiring the anterior placement of the suture and the mesh. This leads to prolonged recovery times, chronic inguinal pain, decreased quality of life, and late return to daily activities. Another popular treatment method is the laparoscopic technique. In this technique, the mesh is placed posteriorly. Posterior placement reduces healing time, pain, and the risk of complications [145].

The possibility of hernia recurrence is lower with synthetic and biological meshes compared to primary suture closure [146,147]. This healing property of the mesh is thought to result from the reduction of tension on facial edges and sutures [148]. Mesh characteristics were found to be effective in the healing process and recurrence of hernia, and therefore, meshes with different characteristics have been developed [149]. In hernia repair, two types of meshes, namely the synthetic and biological ones are used.

Materials such as, polypropylene, polyethylene terephthalate (PET), polytetrafluoroethylene (PTFE), and polyvinylidene-fluoride are used in synthetic mesh construction. The mesh can also be modified by the incorporation of various degradable materials in order to provide ease of use and to prevent intra-abdominal adhesions [150]. In general, polypropylene meshes are the most preferred synthetic meshes in hernia repair [151]. However, there are reports demonstrating that polypropylene meshes induce chronic inflammatory response resulting with foreign body reaction, chronic pain, and decreased quality of life [152,153]. This is due to the amount and structure of the polypropylene, and not being a bioinert material. Late complications such as chronic infection, stercoral fistula formation, and mesh migration were also observed in previous studies limiting their functionality. There is a consensus that synthetic meshes should not be used, particularly in contaminated or infected surgical areas [154].

Human or animal-derived allograft and xenograft biological meshes, including the cell-free dermal matrix and submucosa components, have created a new hope in the surgical treatment of hernias [155]. In particular, biological meshes derived from small intestinal submucosa are of great interest. It has been observed that biological meshes have better physical and mechanical properties, significantly reduce network-related complication rates, and can be used in contaminated or infected surgical areas. After implantation, host fibroblasts proliferate within the meshes, healthy tissue development begins, and finally the entire mesh is absorbed [156]. The main limitations of biological meshes are their unpredictable response at the host and high cost [157]. In addition, the number of studies with biological meshes is very limited and comparative studies with synthetic meshes are rare [158–160]. There is only one clinical study in the literature comparing small intestinal submucosa-derived meshes with a different biological mesh derived from human cell-free dermal matrix [161]. Therefore it is not yet known exactly how the use of biological network will provide advantages in clinical applications.

The basic feature desired for an ideal mesh is to support the muscle integrity, where it is located [162]. In this context, one can speculate that biomaterials with mechanical strength should be used. However, erosion of biomaterial, migration, fistula formation, adhesion, chronic pain, and motion limitation observed in clinical studies have led to the reassessment of biocompatibility features and the development of better biocompatible meshes [150]. In order to minimize complications and increase biofunctionality and

biocompatibility of meshes, low-density and large-porous meshes were first developed in 1998 [152]. Comparative clinical studies have shown that low-density and large-porous (> 1 mm) meshes cause lesser inflammatory response and complication than that of conventional high-density and small porous meshes [163–165].

Anatomical location of hernia and mesh (inguinal, incisional, hiatus, and parastomal hernia repairs, bursting abdomen, etc.), and the applied surgical technique (conventional open, laparoscopic, etc.) are critical parameters while determining the efficacy of the surgery. In this context, it is reasonable to speculate that mesh structures should be specific for each condition [150]. In laparoscopic techniques, mesh is permeable and is expected to induce a low level of adhesion. However, in the open surgical technique, elasticity is extremely important to prevent chronic pain and mesh migration. Therefore, it is necessary to decide the type of mesh to be used prior to the operation, in order to achieve the desired results [166].

There is no generally accepted common mesh for all hernia operations. Although it is known that large-porous meshes cause less inflammatory response than other mesh structures, some complications such as bacterial contamination and mesh migration are still important problems needed to be considered [150]. Therefore, structure and biocompatibility characteristics of existing meshes must be improved in order to reduce the rate of complications.

11.4 Cardiovascular biomaterials

CVDs are the number one cause of human death all over the world. Therefore, biomaterials including coronary stents, heart valves, vascular grafts, pacemakers, and artificial hearts are widely used and investigated to treat and improve the outcomes of CVDs [167,168]. The cardiovascular system is composed of the heart and blood vessels and cardiovascular biomaterials are mostly in contact with arterial and venous blood, vascular endothelial cells, fibroblasts, myocardium, and ECM. Cardiovascular biomaterials are divided into three categories according to their application modes: temporary external devices, temporary internal devices, and permanent internal devices. This classification is significant while determining the type of required biocompatibility testing. Contact duration with blood is also an important parameter while determining the testing parameters. Contact duration is considered limited if it is less than 24 hours, prolonged between 24 hours and 30 days, and permanent if it is longer than 30 days. Biomaterials used in the cardiovascular system mostly include metals, metal alloys, polymers, and some biological materials [167,169]. The main biocompatibility issues related with cardiovascular biomaterials include processes initiating after blood and biomaterial interaction. These processes basically include protein adsorption to the biomaterial surface, platelet aggregation, activation of inflammatory and immunological system, endothelialization, mesenchymal cell infiltration, shear stress, and mechanical factors [170].

11.4.1 Coronary stents

Coronary revascularization procedures including percutaneous coronary interventions and coronary artery bypass surgery have been revolutionized over the years. Thanks

to significant improvements in percutaneous treatment strategies, it has become the preferred revascularization approach compared to surgery in coronary artery disease (CAD) patients [171].

The evolution of percutaneous treatment strategies originally started with balloon angioplasty in 1977 [172]. Although occluded arteries were partially dilated by inflation of the balloon, acute occlusion of the vessel and significant restenosis originating from thrombotic processes, elastic recoil and neointimal hyperplasia were major drawbacks limiting the applicability of this intervention [168]. Therefore implantation of intravascular mechanical support systems made of cylindrical metal scaffolds namely stent were proposed to mitigate the complications of balloon angioplasty, and the first bare metal stent (BMS) was implanted to patients in 1987 [173]. In the subsequent years, BMS has been widely used to treat CAD patients. However, there was an undeniable risk of stent restenosis with BMS implantation originating from significant neointimal hyperplasia after stent deployment, stimulated by the proliferation of smooth muscle cells, monocyte cell infiltration, and proinflammatory processes in the endothelium. Besides, materials of BMS, which have strong support force capability such as medical grade 316L stainless steel, titanium alloy, cobalt alloy, and other metals, are prone to cause immunological rejection in the endothelium after stent deployment, which is also a risk for neointimal hyperplasia [174–176]. In addition to restenosis, there is also a risk for acute and subacute thrombotic occlusion with BMS implantation, which is driven by platelet aggregation and coagulant protein deposition and needs lifelong antithrombotic therapy to prevent [176,177].

To achieve restenosis problem observed with BMS, drug-coated stents namely drug eluting stent (DES) releasing antiproliferative agents such as sirolimus and paclitaxel were developed to inhibit neointimal hyperplasia. Although restenosis rates have dramatically decreased with the implantation of DES, late stent thrombosis, delayed healing, presence of a permanent metal object in the vessel, and prolonged exposure of stent struts to blood flow have been major concerns in the interventional cardiology era [178,179]. These problems have not been adequately solved even with the development of new generation DES designs. Therefore, a new generation of stents named as biodegradable stent (BDS) has been developed and introduced for application in the recent years. Theoretically, BDS is considered to scaffold the vessel wall to prevent acute occlusion and elute an antiproliferative drug to inhibit neointimal hyperplasia in a few months and then degrade. It is absorbed by the body, while allowing the vessel healing and reorganizing adequately. After successful complete degradation, the healed vessel will be free of a foreign object and perform physiological vessel functions. Although FDA approved the first BDS in 2016, clinical trials demonstrated high incidence of device thrombosis and target lesion revascularization compared to DES [168]. Therefore, implantation of BDS is not recommended in routine clinical practice at present but investigations to achieve these problems continue [171].

The goal of discovering fully biocompatible and biofunctional coronary stent has not been achieved until today. The main purpose of coronary stent biocompatibility investigations is to find appropriate biomaterials, which are resistant to thrombotic and inflammatory reactions and help endothelialization of the vessel in addition to providing radial support, become flexible and radiopaque. Initially, BMS was made from surgical grade metal alloys and subsequently evolved to CoCr and platinum alloys, which are not

compatible with the vasculature. They are prone to thrombosis due to their inherent surface characteristics and exert excessive smooth muscle cell proliferation. Stent thrombosis problem can be achieved by antiplatelet therapy, but restenosis still occurs despite adequate medical therapy [174]. Activation of immune response, triggered by undeniable damage to the vessel wall during stent placement, is the major reason for restenosis development. Highly proliferating smooth muscle cells infiltrate into the vessel lumen, secrete ECM components, and subsequently in-stent restenosis develops [180].

DES system mainly includes three components: metal stent, drug carrier, and drug coating. Its development has mainly focused on the failure mechanisms of BMS such as thrombosis and restenosis [175]. The biocompatibility of BMS is related with the stent material and design only, whereas polymers used for coating the stent and drug, and released drug may affect the biocompatibility of DES. Restenosis problem has been partially resolved with the cost of delayed healing and reendothelialization, although thrombogenicity, especially late and very late stent thrombosis, is still a significant problem in DES placement. Therefore, subsequent modifications have also included part of metal alloys, coating polymers, and eluted drug components [174,181]. Nevertheless, a significant proportion of patients still suffers from adverse cardiac events questioning the safety of DES and the need for more biocompatible and biofunctional coronary stents [182].

Another important aspect needs to be considered in the biocompatibility of coronary stents is that they are permanent in the body as a foreign object. Therefore, stents with biodegradable properties have been developed to eliminate deleterious effects of stents on vessel geometry, shear stress, and cell signaling. They confer the advantages of reduction from the adverse effects of permanent material, the removal of the stented vessel's rigid caging capacity, the suitability for future treatment options such as bypass surgery, allowing local drug delivery, relief of anxiety of the implanted foreign material and pediatric applications [183]. They are mainly made of biodegradable polymeric materials or metal alloys. The polymeric materials include polymers synthesized from lactic acid, glycolic acid, and caprolactones. Metallic materials mainly include Fe, Mg, Zn, and the combination of other metals such as Li, Ca, Sr, Mn, etc. [168]. The main issue about the biocompatibility of BDS mostly includes the degradation products of materials. Biopolymer-based materials are generally regarded to be nontoxic and biocompatible. They are less likely to cause significant chronic inflammatory reactions. Their degradation products can be eliminated from the human body by enzymatic and hydrolytic processes, leading to the release of final low molecular weight degradation products. Foreign body giant cells and leukocytes can eliminate these final products [184,185]. On the other hand, the released metallic ions from degraded metallic stents may induce both local and systemic toxicity. Due to the fact that, all of the alloyed elements will enter into the body, biosafety and biocompatibility of other alloys need to be considered. Basically, alloy materials should compromise essential metallic elements, which are metabolized by the body [186,187]. In addition to biocompatibility and biosafety issues, current BDSs require improvements in terms of mechanical features, strut thickness, biodegradation characteristics, inflammatory responses, thrombosis, restenosis and drug eluting properties [168]. At present, BDS performance is suboptimal and not suggested in clinical practice but it is likely that it will be the main choice of coronary stent applications in the near future with the help of future studies.

11.4.2 Heart valves

The frequency of heart valve replacement operations increases each year with the aging population all over the world [188]. Due to increasing demand, artificial heart valves are widely used. There are two types of artificial heart valves at clinical markets at present: mechanical and biological valves. Mechanical heart valves are engineered from synthetic materials such as metals, ceramics, and polymers. Biological heart valves are made of biological tissues after proper physical and chemical modifications including homografts such as cadavers or patient's own valve (Ross procedure), and xenografts such as porcine aortic valve and bovine pericardium. The main goal of valve replacement is to provide an easily implantable and biofunctional solution to increase blood flow through the valve, and preserving physiological heart functions, while decreasing the risk of complications related with biocompatibility. Each type of valve has many advantages and disadvantages varying between individual patient populations [189].

The history of heart valve replacement dates back to almost 60 years ago and their development has been associated with the identification and appropriate utilization of biocompatible, mostly blood compatible materials. Therefore, a set of requirements has been developed over the years for a material to be used safely for valve replacement. Valve material must cause minimal trauma to the surrounding milieu including blood elements and endothelial tissue. It must have good resistance properties to mechanical and structural wear, minimizing the risk for platelet and thrombus aggregation. It must not be degradable in the physiological milieu and it must not absorb blood contents and release unwanted substances into the blood. In addition, material used for valve replacement must be suitable for sterilization and should have acceptable surface characteristics [190].

Mechanical valves have evolved from ball and cage type to tilting disc and subsequently to bileaflet valves, respectively. The modification of these valves has revolutionized through their geometric designs, hinge mechanisms, and applied materials. When implanted into a patient with a good surgical technique, they are durable and show long-term functional capacity. However, they are very prone to platelet aggregation, thrombus deposition, and subsequently cause embolic complications. Therefore, patients with mechanical heart valves need to be on lifelong anticoagulation therapy. For this reason, they are not the preferred choice of therapy in elderly patients and pregnant women. In addition, hemolysis of red blood cells due to the shear stress between blood and valve surface is a significant problem affecting the biocompatibility of mechanical valves. Microbial contamination risk, named as infective endocarditis, is another condition limiting their use, although this limitation is also obvious in biological heart valves [167,170,189,190].

Similar to the evolution process of mechanical valves, biological heart valves have also gone through varying modifications since the 1960s. The high thrombogenic potential of mechanical valves has led to an increasing use of biological valves in the recent years, due to the fact that biological valves do not possess thrombogenicity risk and for this reason they do not require lifelong anticoagulation. At the present, porcine aortic valves and calf pericardium are the most preferred biological materials for valve replacement. The most significant biocompatibility problems of biological valves include immunological reactions and time-dependent structural changes on the valve such as calcification and leaflet wear.

Because of their limited durability, they are not recommended for replacement in children and young adults [5,189].

Biological valves undergo various modifications to remove or inactivate resident cell antigens and nucleic acid residues to avoid the immunological rejection risk. For this purpose, tissues may be decellularized by detergents, enzymes, and chemical agents. One important point that needs to be considered during this process is that decellularization procedure should not alter the architecture, ultrastructure, mechanical integrity, and biological activity of the remaining ECM [188]. Biological valves are usually fixed in glutaraldehyde solution to reduce immunogenicity, infection risk, and increase resistance to degradation of collagen either enzymatically or chemically. However, there are concerns about the cytotoxicity risk of glutaraldehyde by leaching out the valve and its potential to enhance calcification [191,192]. To reduce the risk of valve calcification, antimineralizing treatments such as alpha-oleic acid or surfactants are used. These agents bind to the tissue covalently and prevent calcium influx [189]. The demand for better performance with biological heart valves has led to the development of stentless valves, which has been supposed to reduce stenosis and improve the hemodynamic performance of the valve by lacking the stent covering the valve and sewing cuff. The absence of these parts may minimize the residual stenosis and provide implantation of a larger valve, resulting with enhanced hemodynamics and clinical effect [193].

The evident and diverse biocompatibility problems observed with mechanical and biological valves have guided the researchers, clinicians, and operators to alternative solutions. Therefore, surgical techniques to repair the diseased valve have been developed instead of implanting a new valve. In addition, percutaneous transcatheter valve replacement strategies have been introduced and are in their infancy holding great promise for the future [194].

11.4.3 Implantable pacemakers

Cardiac pacemakers are smart electronic devices implanted to fix the cardiac arrhythmias. In some situations, pacemakers can defibrillate the heart and improve the pumping function of the weak heart muscles by synchronizing the heartbeats. They include a lithium battery, generator, and leads connecting the generator to the heart. They are classified as temporary or permanent according to the duration of usage. If there is an arrhythmia occurring due to an emergency situation, temporary pacemaker is implanted through the veins to protect the patient from the detrimental consequences of arrhythmia. The generator of the temporary pacemaker is placed outside the patient's body and only used in hospitals. In contrast, the permanent pacemaker is implanted into the chest of the patient for long-term use. Patient with a permanent pacemaker lives with the pacemaker for the rest of his life [195].

The generator and battery of the pacemaker, which consist the active components of the device, are encapsulated in a hermetically sealed titanium can, which is resistant to corrosion. Leads carry pulses for both sensing and pacing function between the generator and the heart. They are thin and insulated wires, which must be resistant to fracture and corrosion. Therefore, platinum group metal alloys or CoCr are used for leads accompanying insulating sleeve covering the lead [5,196]. Polyurethanes and silicones have also been

widely used to insulate the pacemaker leads for their acceptable physiochemical and mechanical characteristics. Besides, polyurethane has excellent biocompatibility features allowing unlimited usage in blood contacting devices, in addition to having high shear strength, elasticity, transparency, and resistance to infectious agents and thrombus formation [5,167].

The main problem in cardiac pacemaker devices is the high infection rates, which are associated with higher morbidity, mortality, and economic costs despite the increasing surgical experience. It is stated that the rate of cardiac pacemaker infection has been higher than the number of implantations in the recent years. To prevent infection development on pacemakers, antibiotic and antiseptic agents are widely used [197]. In addition, due to the presence of ferromagnetic components in pacemakers, MRI is contraindicated in these patients. MRI conditional pacemakers are to overcome this problem and implanted in the clinics [198]. Moreover, investigations about the development of leadless pacemakers may overcome the biocompatibility and biofunctionality issues regarding infections, lead complications, MRI imaging obstacles, and procedure related difficulties [195,199].

11.4.4 Left ventricular assist devices

Advanced stage heart failure is a significant health problem with very high mortality rates. Treatment options are very limited in this patient group and cardiac transplantation is the only exact solution, although it does not satisfy the need because of the shortage of donor numbers. Therefore left ventricular assist devices (LVADs) were developed in the 1960s and introduced into the clinics to replace the function of the failing heart. At first, it emerged as a bridge-to-transplantation therapy but thanks to huge progresses in device technology and clinical management strategies, LVADs are now used for bridge-to-candidacy and bridge-to-recovery therapies [200].

Although there was a significant improvement in the course of heart failure with the first-generation LVADs, they had several limitations including their large size and pulsatile flow characteristics causing a loud sound. Therefore, second-generation and subsequently third-generation devices, which are smaller, more durable with continuous flow characteristics and more biocompatible, have been introduced into clinics. The advancements in LVADs have revolutionized the course of heart failure. However, there are still limitations of this treatment modality, which need to be improved in terms of biocompatibility [201,202].

Right heart failure, infections, neurological complications, thrombosis, and bleeding are the major mortality drivers in LVAD implanted patients. Unloading of the left ventricle after the implantation of continuous flow LVADs may cause right ventricular dysfunction and pulmonary hypertension through an imbalance between left and right ventricles resulting with right heart failure. The incidence of infection during LVAD implantation varies but has gradually decreased in the recent years with the use of antibiotic therapies. Neurological complications represent the most debilitating complications, which may be associated with several factors such as patients' clinical conditions, metabolic conditions, heart pressures and dimensions, arrhythmia episodes, anticoagulation levels, and infection status. Thrombosis and bleeding might also be associated with the

anticoagulation levels and are serious complications causing mortality in this patient group [200–202].

The biocompatibility issues of LVADs are generally related to surface features, interaction with the blood, activation of the coagulation cascade, anticoagulation, inflammatory cell adhesion, coatings, flow characteristics, and shear stress. It is known that significant platelet, leukocyte, and red blood cell activation occur with the use of centrifugal pumps and rotablation in LVADs resulting with thrombosis and hemolysis [203,204]. Strategies to appropriately maintain pumping volume with optimal fluid dynamics are important to hinder hemolysis and thrombosis [205]. Anticoagulation regimes can reduce thrombosis risk with the cost of bleeding risk. Titanium and its alloys are used to manufacture LVADs because of their hemocompatibility, tissue-compatibility, and low cost. To reduce the risk of thrombosis, bleeding and infection, surface coatings for LVADs have been investigated. These surface coatings include titanium nitride coatings, diamond-like carbon coatings, 2-methacryloxyethyl phosphorylcholine coatings, heparin coatings, textured surfaces, and endothelial cell lining. Titanium nitride coatings and textured surfaces are the most applied coating procedures to improve biocompatibility and hemocompatibility of LVADs, while cell-based coatings such as endothelial cell lining are still in its infancy [206].

11.4.5 Vascular grafts

The history of vascular surgery dates back to the last decades of the 19th century, when Eck anastomosed portal vein to the inferior vena cava with fine silk sutures. However, vascular grafts have been in surgical use since the middle of the 20th century. The first application of autologous vascular grafts in bypass operations started at these times. Meanwhile, one of the first synthetic vascular grafts, namely Vinyon-N cloth, a nylon derivative, was developed but failed to be stable and durable. Similar to the nylon, materials such as metal, glass, and ivory have failed to be an ideal constituent for vascular grafts. Failure of these materials has drawn attention to two major biocompatibility issues regarding synthetic vascular grafts over the years: thrombogenicity and durability. Intimal hyperplasia and poor hemodynamic features have also been significant problems regarding biocompatibility [207,208].

In general, an ideal vascular graft must have some features to fulfill the body's mechanical and cellular needs. Above all, a vascular graft needs to be biocompatible, nontoxic, nonthrombogenic, and resistant to infection with a proven high long-term patency. It must cause an acceptable healing response in the body without inflammation, hyperplasia, or fibrous capsule formation. It must have adequate porosity to allow for the growth of the autologous tissue. It must have sufficient mechanical strength and the ability to cope with the hemodynamic stress without failure similar to the native vessel. It must be resistant to creep and deformation, which may cause aneurysm formation in a long time, and express physiological characteristics such as vasoconstriction and relaxation similar to a native vessel. In addition, an ideal vascular graft must be easily and economically manufactured, produced, sterilized and stored off-the-shelf in various sizes. During the operation, it must be flexible, resistant to kinking, and easy for surgeons to handle with acceptable suture retention [208–210]. Despite intensive studies in the material era, the goal of designing a vascular graft providing all of these criteria has not been achieved yet.

Autologous grafts including internal mammary artery and saphenous vein are usually used during coronary artery bypass surgery with acceptable patency rates. However, in some conditions such as previous bypass surgery or peripheral artery disease, autologous grafts may not be a viable option. Therefore, synthetic grafts are usually used as existing alternative synthetic vessels. These alternative synthetic grafts mostly include PET named as Dacron and expanded PTFE (e-PTFE) in clinical practice [207,208]. Dacron grafts are manufactured in woven or knitted forms. Woven form has smaller pores, which decreases blood leakage and makes it more efficient compared to the knitted form. Dacron grafts can also be coated with protein such as albumin or collagen to prevent blood loss and to be more biocompatible [167,211]. Dacron grafts are durable and can have patency rates for more than 10 years without significant alteration. However, they are prone to dilatation in arterial environment because of its fabrication technique [212]. e-PTFE grafts, which are formed by the chemical and physical modification of PTFE namely Teflon, are used for small diameter arteries (<6 mm) with lower flow rates. They have low thrombogenicity, restenosis and calcification rates. In addition, they show biochemically inert characteristics [167]. Therefore, e-PTFE is used in small diameter arteries below aortic bifurcations, whereas PTFE is successfully used in large diameter arteries with high flow such as aorta. PTFE is also used in prosthetic heart valve rings. Nevertheless, their patency rates significantly decrease in low-flow, small diameter arteries, which limits their wider surgical usage [167,207,213]. PTFE is very resistant to chemical substances, microorganisms, and fungi. It is physiologically inert, nontoxic, and does not induce allergic reactions. Besides, it can be sterilized easily and it is resistant to friction and the effect of foreign substances [213].

Vascular grafts used during surgery either autologous or synthetic are nondegradable and they are permanent in the body, which make them susceptible to adverse foreign body responses including blood and tissue reactions as well as microbial contamination. Foreign body reactions start immediately after implantation of graft to the body. The first step involves protein adsorption to material, which is known as Vroman effect. This process is followed by platelet adhesion, inflammatory cell infiltration, and migration of endothelial and smooth muscle cells [170,208]. Thrombosis is the main pathophysiological mechanism causing acute graft failure at the first days of implantation. However, in the long term, neointimal hyperplasia plays a role in graft failure, a process contributing to atherosclerosis. Neointimal hyperplasia is the remodeling of the vessels in which myofibroblasts and smooth muscle cells migrate to the intima, proliferating extensively and depositing ECM elements forming a neointima [214,215]. Basically, it consists of five steps involving platelet activation, inflammation, and related cells' deposition, coagulation, vascular smooth muscle cell migration, and proliferation. Moreover, fibroblast transdifferentiation to myofibroblast, and their migration to intima from adventitia are involved in the pathophysiological mechanism of neointimal hyperplasia [216,217].

11.5 Conclusion

Biocompatibility, which refers to the properties of a biomaterial to be used safely in a living system, has evolved in the 20th century. It is closely related to a principle of

Hippocratic Oath, namely “Primum Nihil Nocere,” meaning that first of all, do not harm. This is the main principle in medicine. However, our current understanding about biocompatibility of biomaterials corresponds much more of this adage defined centuries ago. It is the capability of a biomaterial performing appropriate function in the organism without inducing an unacceptable harm. Moreover, it is not only related with the lack of toxicity, but also associated with biofunctionality and all adverse effects, where the material is applied. At this point, one can suggest that an ideal biomaterial should be inert and do no harm in the body after implantation. In fact, inertness was regarded as a synonym of biocompatibility at first until the recognition that every material evokes a kind of response in the body regardless of its physical and chemical features or desired function. From a clinical point of view, this response constituting biocompatibility may vary according to patient characteristics including age, sex, and comorbid situations. In addition, it should be kept in mind that the type of surgery and experience of the surgeon may influence this process significantly. Therefore, biocompatibility definition is not only associated with the type of material, but also related with the applied patient and the surgeon performing the procedure. In this context, the main issue needs to be discussed is whether it will be possible to create fully biocompatible and biofunctional materials, which do not evoke any response in the body. We believe that this goal will be achieved in the future with the help of prospective developments in biotechnology, regenerative medicine, and personalized medicinal sciences.

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Standardization and regulation of biomaterials

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12.1 Introduction

Biomedical materials or biomaterials can be defined as “a substance that has been engineered for guiding or controlling the biological interactions within a living system during therapeutic, regenerative, or diagnostic purposes to maintain or improve the quality of life of the individual”. Biomaterials differ from other classes of materials due to their ability to maintain their function in a biological system for the intended period without damaging the whole system [1,2].

Generally speaking, the biomaterials can be classified into four groups according to their purposes;

1. Functional rehabilitation: i.e., synthetic heart valve, artificial joint, dental implant.
2. Therapeutic/healing purpose: i.e., suture, wound dressing, local drug delivery system.
3. Regenerative purpose: i.e., scaffold, hydrogel, cell, and growth factor.
4. Diagnostic purpose: i.e., tumor imaging by nanoparticle, monitoring blood sugar by the biosensor.

Biomaterials play a critical role nowadays in medical, dental, pharmaceutical, and esthetic fields. In fact, in modern times, the field of biomaterials has involved medicine, dentistry, physics, chemistry, biology, engineering, and information technology (IT). Researchers and experts from these fields are being involved in the improvement and advancement of biomaterials through innovations. However, only a few of the new biomaterials are able to pass the challenging regulatory policies to enter the translation and commercialization phases. Even though a close long-term follow up is required to monitor possible delayed adverse effects these in turn affect the biomaterials marketing policies. This can be seen as some commercialized biomaterials have been banned several years

after their service availability due to recorded adverse effects or complications in the long run.

In this context, the importance of biomaterials' standardization and regulation should be highlighted. Ideally, the biomaterial studies should be able to analyze the biomaterials from the health-related risk/benefit factor (short-term and long-term), economic, applicability, availability, affordability, and patient compliance. Furthermore, there should be strategies for standardizing the research outputs with regards to the overall study design, research methodology, interpretation and reporting of study results, and conflict of interest. Through this chapter, we highlighted the relevant topics regarding biomaterials' standardization and regulation.

12.2 Biomaterials for therapeutic and regenerative medicine

12.2.1 Biomaterial design, fabrication, characterization, and documentation

Biomaterials research usually follows a common path starting from biomaterial designing and production, biocompatibility analysis, in vitro cell behavior study, animal model testing, and possible human trials. The different protocols should be considered at each phase with respect to available standards and regulations [3]. There are different guidelines for evaluation of material properties according to national or international standards such as: International Organization for Standardization (ISO), American Society for Testing of Materials (ASTM), Association for the Advancement of Medical Instrumentation, United States or European Pharmacopoeia, and National Institute of Standards and Technology. Furthermore, a variety of basic requirements have been defined for biomaterial performances in some fields such as for calcium phosphate coatings on orthopedic and dental implants [4].

The details of biomaterials' study design and production techniques should be reported properly to address the issue of reproducibility. This is very critical since the technique that cannot be reproduced should not be expected to result in similar biomaterial products. A close analysis of available literature would reveal the general reports on biomaterials production methodologies; however, the minor critical technical details are frequently neglected and not reported. This would result in questionable reproducibility which is considered a critical study limitation or shortcoming. Furthermore, it is known that the technical details of biomaterial processing can impact the biomaterials' properties. For example, the calcium phosphate bioceramics with similar chemical composition processed under different sintering conditions could produce different physicochemical and biological behaviors [5].

Another important issue is related to biomaterials' characterization methods in biomaterials study (Table 12.1). A full report of basic and advanced physicochemical properties of biomaterials would facilitate a better study planning based on more realistic predictability of biomaterial functions. It is reported that biomaterials with comparable physicochemical properties would behave differently under similar biological conditions due to their unreported minor differences [6–8]. Therefore, a report of appropriate details including exact biomaterial geometry, particle size, grain size, crystal size and shape, surface charge, and

TABLE 12.1 Parameters that should be considered during biomaterials production.

Parameter	Recommendation
Synthesis/processing	- Ratio calculation methods during biomaterial synthesis or processing should be clearly explained (v/v%, w/v%) - Name and detail of additives (i.e., preservative, binders, emulsifier, plasticizer) and preparation, dilution, mixing methods should be stated
Phase purity	- FTIR, full crystallography (XRD), and morphological analysis (TEM, SEM) are critical - Presence of minute impurities or secondary phases should be excluded which may trigger certain biological responses
Sintering/calcination program	Detail of full sintering program should be reported; i.e., sintering temperature, sintering environment, heating rate, soaking time, cooling rate, name and model of sintering machine and its calibration policy
Porosimetry	- Analysis of porosity parameters should be performed carefully with adequate technical details including analysis of total porosity, pore size, pore geometry, pore distribution pattern, and interconnected porosity - Pore measurement methodology should be reported - The rationale for the selection of pore size should be stated - Correct terminology should be used; i.e., macropore, micropore, mesopore, and nanopore
Particle size/shape	Particle/grain size and shape should be evaluated and reported using proper terminology
Mechanical properties	- Full mechanical properties should be reported; i.e., mechanical strength, tensile strength, toughness, and Young's modulus - The testing condition should be clear; i.e., wet or dry, room temperature - Testing parameters should be elaborated - The intended/expected mechanical performances of biomaterials within a living system should be explained
Biodegradation	The biodegradation and dissolution properties of biomaterials should be tested appropriately
Permeability	The fluid flow through the biomaterials should be analyzed; i.e., the study of swelling ratio, water uptake percentage, water contact angle, and hydrophilicity
Manufacturability	- Ease of biomaterials production, processability, and manipulability should be reported - The best manipulability and handling method should be recommended
Sterilization and storage	- The sterilization or disinfection method should be reported - Storage condition and timing should be addressed - Possible biomaterial changes due to sterilization techniques or storage should be discussed - In general, a biomaterial should maintain its nature during bioprocessing, disinfection, and sterilization process
Statistical analysis	In all testing techniques; the testing condition, sample preparation, sample size, sample selection method, statistical methods, and level of significance (<i>P</i> value) should be reported

FTIR, Functional group analysis; *SEM*, Scanning electron microscopy; *TEM*, Transmission electron microscopy; *XRD*, X-ray diffractometer.

spatial orientation can provide a clear view of impact of these factors on physcobiological properties [9]. Unfortunately, our review of the literature has revealed the lack of proper characterization of bioceramics before their applications in animal studies [10].

Earlier, we addressed the problem with the inconsistency of using terminologies in explaining the porosity dimension of biomaterials. For many authors, the definition of

such terminologies is confusing in literature as there are no commonly accepted definition criteria in this context. As such the terminologies such as nanoporosity, mesoporosity, microporosity, and macroporosity are applied randomly according to the author's understating of such classification [11–13]. Another problem is related to the failure of researchers to apply the available commonly accepted terminologies. In this context, it is very common to come across papers that talk about “nano” biomaterials while the actual dimension of their biomaterials exceeds 100 nanometers [14].

Each lot of biomaterials should be sterilized before cell or animal studies considering the possible impact of sterilization methods on biomaterial properties [15,16]. As such, it is suggested to do the required biomaterial characterizations after sterilization. The guideline for sterilization and disinfection in healthcare facilities by Centers for Disease Control and Prevention (CDC) is a well-prepared standard to stick with [17,18]. The impact of storage on biomaterial properties should also be addressed, this includes decomposition, denaturation, or phase changes. In general, it is recommended to reduce the storage time and standardize the storage condition of biomaterials before applications.

Here, we suggest the basic testing methods during biomaterials' synthesis or preparation (Table 12.2). Adherence to these parameters as well as other international standards would reduce the chance of bias in biomaterials analysis and produce a platform that facilitates the comparison of the available literature that in turn helps expand the field of biomaterials.

12.2.2 In vitro cellular response analysis for biomaterials study

Cytotoxicity and biocompatibility analysis is the first critical step after the production of new biomaterials before further in vitro, in vivo, or in situ studies. With new innovations in biotechnology and the introduction of complex biomaterials, the traditional concept of biocompatibility has been challenged. As such, the updated guidelines are lacking with regards to current stem cell-based therapeutic or regenerative studies.

Following the biocompatibility and cytotoxicity clearance, there are some additional general criteria for the expected biological performances of certain biomaterials for the intended application. For example, with regards to bone tissue engineering, the biomaterials are classified to be osteoconductive, osteoinductive, or osteogenic. Bioactivity is a commonly used term based on the biomaterials' performances in a living system. However, this term is too general and does not seem to be a perfect fit to define biomaterials that positively benefit the living system. It is a general term that only explains the biological performances of biomaterials and nothing more. In fact, biomaterials that interact with the body resulting in short-term or long-term adverse effects could also be defined as bioactive regardless of their negative performances.

An additional problem is related to challenges in comparing or contrasting stem cell studies due to conflicting reports in the literature. For instance, there are controversial findings related to phenotypic characteristics and antigen expression potential of stem cells. This is mostly attributed to the absence of universal guidelines and failure to adhere to available standards. Furthermore, attention should be made toward the fact that minor

TABLE 12.2 General recommendations for characterization techniques of biomaterials.

Test	Parameters	Standard unit
XRD	- Crystallography according to the ICDD database - Type of machine and testing conditions should be reported - Follow the radiation guidelines for each biomaterial (theta degrees, kV, mA)	Intensity/ 2θ degree
PSA	Analysis of volume-based particle size distribution pattern using laser diffraction method	μm , nm
SEM	- Microstructural study for evaluation of surface texture, morphology, particle/crystal/grain size, porosity, and roughness - Follow the recommended testing parameters	μm , nm magnifications, i.e., $\times 100$, $\times 500$, $\times 1000$
MT	- Study of mechanical properties including; compressive strength, tensile strength, toughness, fracture resistance, etc. - Follow the available guidelines (i.e., ASTM) - Report the testing condition; i.e., wet/dry, static/dynamic, loading cells, rate, pattern, etc.	kPa, MPa, N
FTIR	- Chemical/structural analysis of biomaterials - Follow the recommended guidelines for each biomaterial (i.e., wave number cm^{-1})	Wave number cm^{-1}
TEM	Analysis of morphological/spatial configuration and crystalline diffraction pattern	nm
EDS/XRF	- Elemental chemical analysis. - Study of elemental ratio (i.e., Ca/P molar ratio).	$K\alpha$, keV, nm
Porosimetry	- Study of porosity parameters of biomaterials by gas sorption, mercury intrusion, electroacoustic spectroscopy, capillary flow porosimetry, and CT-scan - Measuring specific surface area, specific pore volume, pore size distribution pattern, etc.	P.U. $\cdot\varphi\theta$
Micro-CT	Analysis of internal biomaterials microstructure by 3D reconstructed images	μm /voxel

EDS, Energy dispersive X-ray spectroscopy; FTIR, Fourier transform infrared spectroscopy; ICDD, The International Centre for Diffraction Data; Micro-CT, micro-computed tomography; MT, mechanical testing; P.U., porosity unit; PSA, particle size analyzer; SEM, scanning electron microscopy; TEM, transmission electron microscopy; XRD, X-ray diffractometer; XRF, X-ray fluorescence analysis; θ , contact angle; φ , porosity.

differences in study design or research protocol would have a tremendous impact on the performances of stem cells. For example, differences in donor source, isolation techniques, passaging, and culture conditions are reported to influence the expression pattern and differentiation profile of stem cells [19–22]. Stem cell-related studies should properly address the exact source of stem cells (i.e., species, gender, age, anatomical site, health status), isolation methods, culturing condition, and characterization techniques.

We have discussed the fact that the controversies in current literature indicate the need for the development of new standards in line with the current innovation or to update the traditional protocols in isolation, culture, and characterization of stem cells [23]. Furthermore, adherence to some basic guidelines would decrease the bias by reducing the confounding factors that ultimately result in more homogeneous reporting. For studies that explore stem cell-biomaterials interactions, the biomaterials should be characterized

as discussed earlier. Further incorporation of signaling molecules should be well documented which may include genes, growth factors, proteins, and drugs.

12.2.3 In vivo animal model for biomaterials study

An animal study is a perfect model to analyze the biological performances of biomaterials in a 3D environment. Due to the complexity of the biological model and interferences of multiple factors, biomaterials screening for therapeutic purposes, safety-efficacy issues, and studies of disease pathogenesis may not be practical using in vitro research. Difficulties with ethical approval and economic limitation have a considerable impact on the conduction of animal studies [24,25]. However, all new biomaterials uneventfully require a trial in human models due to the fact that even an animal model is a poor predictor of performance in humans.

Due to difficulties encountered during animal studies (i.e., ethical approval, budget limitation), it is much advised to carefully design the study protocol (Table 12.3). This may require a comprehensive literature review to select the best animal model according to the type of biomaterials. Ideally, the animal model should have the following properties [26]: similarity to human genome such as in larger animals, high reproducibility, low morbidity

TABLE 12.3 Suggested parameters to be investigated during biomaterials studies in the animal model.

Parameters	Recommendations
Type of animal	• Selection of large animal models would better reflect human biology • Animal species, sex, age, and weight should be reported • Ethical approval should be mentioned
Sample size	• A minimum number of animals allowing statistical conclusion is required • A statement on sample size calculation and justification is important • Effect size, SD, type I error, power, and attrition rate to be reported
Anatomical site	• Anatomical site and the selection criteria should be defined • Expected nature of the functional load, intensity, duration should be reported
Housing and husbandry conditions	• Commonly neglected factor that should be noted to address the bias of confounding factors in reported findings
Experimental surgical procedure	• Surgical technique, duration, instrument/materials used need to be reported • Preservation or interferences of the periosteum layer should be noted • Type of the bone (D1–D4) and remodeling capacity should be evaluated
Defect dimension	• Accurate bony defect dimension should be reported • CSD should be defined based on the animal model and the nature of the study • ASTM standard guideline (F2721-09) for CSD should be noted • An untreated empty defect should be used as a control group
Analysis time	• Analysis interval and rationale should be reported
Total study period	• The rationale for the study period needs to be reported • Screening and optimization of biomaterials may need a short study • Analysis of detailed biomaterials—cell interactions and bone remodeling require longer follow-up (> 6 months)
Investigation types	• Analysis of biological performances of biomaterials may include; histologic, histomorphometric, and radiograph studies (X-ray, CT scan, and MRI)

CSD, Critical size defect; D, bone density measure; MRI, magnetic resonance imaging; SD, standard deviations.

and mortality rate, easy husbandry, low cost, and an adequate area of interest/research area for study and multiple investigations.

However, it may not be possible to have ideal animal models for biomaterial studies, hence, the majority of biomaterial research starts with rodent small animal models (i.e., rat, mice, rabbit) due to budget limitation and unpredictability related to biomaterials' performances [27,28]. Furthermore, there are no generally accepted criteria for the selection of the anatomical site where the biomaterials need to be implanted in the animal model. For example, for bone repair potential of biomaterials, various sites have been selected including femora, tibia, spine, mandible, maxilla, and calvarium. The selection of anatomical site should be according to the purpose of biomaterials, as such, the selection of calvaria as an implantation site is not relevant for biomaterials that are intended for load-bearing applications. Furthermore, there are different types of functional load in a living system that should be considered as well. Biomaterials could be subjected to a different type, direction, and intensity of load at different anatomical sites.

The duration of animal study is another important fact that should be considered. The duration of the study should be determined based on the main objectives of the study, available budget, type of animal, and, nature of biomaterials. In general, a study period of more than 3 months is recommended for histological confirmation of bone regeneration (ASTM-F2721-09) [29].

In general, a well-planned experimental design should take into account all possible study variables as well as potential confounding factors that may introduce bias and affect the internal validity of the overall study. This would also help to better extrapolate the animal studies to human trials considering the study design. Hence, better reproducibility and generalizability of the animal study results can be expected. Of course, due to basic differences between human and animal models, some adaptations or adjustments of study design would be acceptable during clinical trials in humans. However, the main challenge in the transition from the animal model to human trials remains the fact that the findings of animal studies cannot reliably predict the human response [29].

In summary, in order to help a smooth transition from basic studies to human clinical trials, adherence to available standards is highly recommended to reduce the bias and improve the quality of the research papers. For this purpose, several guidelines are available such as Cochrane guidelines [30] and ARRIVE guidelines for improving the quality of research and reporting [31].

12.3 Discussion

12.3.1 Standardization of experimental protocols

The multidisciplinary nature of the biomaterial field resulted in a remarkable degree of discrepancy and controversy in the literature. Almost every research paper uses its own standards and protocols. This has impeded the real translation of available innovations [10]. The progress in the field of biomaterials can be remarkably accelerated through standardized study protocols that allow comparison and sharing of the reported data. Research output should adhere to a set of minimal, basic, and common principles that

would reduce the level of reported controversies among similar studies. Fortunately, there are standards such as that are set by ASTM (F04.04) on biomaterials and tissue engineering products. However, the main problem in this context is two-sided. One side of the problem is related to the lack of generalized standard guidelines in many aspects related to biomedical engineering as was discussed. The other side is the lack of adaptation of these guidelines by academic researchers in the field during research and development. In fact, every laboratory or research center has its own experimental protocol that usually remains unpublished due to confidentiality issues. It is not suggested to disregard the confidentiality and copyright issues or to diminish creativity, but simply to adhere to some minimum standards that facilitate data sharing and speed up the progress in the field and the translation that finally serves those patients in need.

Adherence to available standards initiates a common scientific language that is economically, ecologically, logistically, and legally practical. This is because the available standards (i.e., ISO, ASTM) are set by volunteer teams who work in different industries including manufacturers, regulatory bodies, medical/dental/pharmaceutical associations, research centers, regional/national agencies, and academic representatives. Although, there is a critical need for a global platform to support standardization of research activities worldwide through a common data-sharing platform, there will be many unavoidable challenges to address as this platform is expanding such as: data mining, copyrights, data confidentiality, intellectual property, project management, and business-related issues. These complexities may be even more challenging than setting the first platform, however, this is just minimum pay for a better future [32].

12.3.2 Biomaterial regulations and policies

Today the ultimate goal of multidisciplinary research activities on biomaterials is the development of biomaterials for promoting regeneration of human tissues or tissue alternatives. Although there are many criteria for biomaterial selection; safety, efficacy, and quality are the most important ones according to the regulations provided by legal authorities. For example, human tissue engineering products are subjected to certain nonspecific regulations by good manufacturing practice and good tissue practice in the European Union and the United States. With regards to biomaterials, the risk assessment is a mandatory step that may induce several changes during the developmental phases of biomaterials to fulfill the quality and safety assurance [3].

The quality and safety assessment of biomaterials is not a straightforward procedure, rather they are getting more complex and uncertain with the ongoing emergence of new biotechnologies [33]. Of particular importance is the use of biomaterials for tissue engineering. For example, different scaffolding biomaterials have been produced as regenerative means for restoring defected tissue/body functions. Today, these biomaterials are designed with smart integrities that regulate the selected target cell performances through biophysical, biochemical, and molecular simulations [34]. Further challenges in biomaterials' complexity are now the development of hybrid, hydrogel, or combination biomaterials using the bottom-up manufacturing approach which triggers more specific biological performances. Therefore, the traditional biological evaluation of new medical devices

according to ISO 10993 cannot provide a proper platform to assess safety and efficacy because it cannot predict possible stimulation of target cells in a tissue engineering application. It should be also noted that previous Food and Drug Administration (FDA) approval of a medical device or biomaterial is not a reasonable justification for the development of a new one as even a minor alteration of the component would trigger different biological consequences in tissue engineering. As such, the traditional standards for safety and risk assessment need to be frequently updated to address all possibilities and challenges for translation. This may require new definitions of biocompatibility and bioactivity theories [2,33]. In this context, there are some regulations to address new advancements in tissue-engineered medical products set by FDA Center for Devices and Radiological Health (CDRH) and European Advanced Therapy Medicinal Products (ATMPs).

Regulation policies set by the FDA, European Medicines Agency (EMA), and other regulatory bodies have a remarkable impact on the translation of biotechnology from the laboratory to broad clinical applications. In fact, despite the significant cutting-edge innovations in the field of medicine, the real clinical translations lag much behind the current achievements. Due to several challenges, the regulation policies are stalling the translation despite the fact that there are many biomaterials and techniques with evidence of efficacy and acceptable market. Therefore there is a clear imbalance in the translation phase between the available medical innovations and real patient benefit [35]. Furthermore, there are many national or regional policies that cannot be applied at the

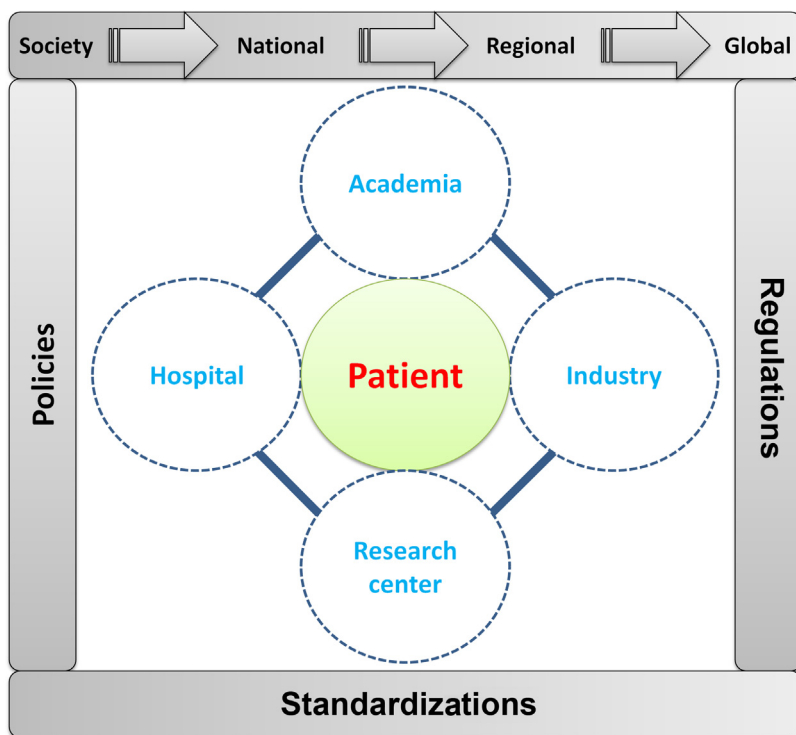


FIGURE 12.1 For the benefit of the patient as the center of the healthcare system, a team approach is essential with frequent communication between hospital, academia, research center, and industry. Standardization should be the base for all research activities, however, the healthcare system as a whole is restricted by regulations and policies that are greatly influenced by differences at society, national, regional, and global levels.

international level due to diversities in societies based on their economic, religious, and cultural background [36,37] (Fig. 12.1).

It should be noted that the regulatory system is influenced by a geographical factor based on the demographic status of the people. This is in turn intimately linked to health economics which impact the budget assignment and prioritization. For example, the health demands in underdeveloped or developing countries could be the control and treatment of infectious diseases while in the developed countries the health demand increasingly moves toward new therapies for degenerative diseases due to increasing longevity. Therefore, some differences between regulations among these countries are acceptable [38].

Religious or cultural factors would also influence the acceptance of certain types of treatments such as donated organs, an allograft from a human cadaver, or xenograft from animal species. For example, Muslims, Hinduism, and Judaism adhere strictly to the “halal,” “sattva,” and “kosher” concepts, respectively. We have discussed the impact of patients’ religious background on the selection and application of biomaterials elsewhere, a fact that has been largely neglected [37]. However, the available challenges that hinder the development of acceptable universal standards for production and translations of biomaterials are in dire need of identification for the ultimate goal of patient benefit.

Along with the regulatory issues, the manufacturing issues have a major impact on the successful clinical translation of innovations balancing between ethical policies and overwhelming clinical needs. Establishing a new manufacturing process and industrial marketing in tissue engineering requires proper investment and business modeling to address clinical needs and opportunities in global commercialization. Therefore, the translations in tissue engineering need to consider a proper manufacturing paradigm to address cost-effectiveness and time management for bulk production and commercialization through phase 1 to phase 3 clinical trials [39]. We have addressed earlier the impacts of ecologic, economic, and time factors in biomaterial engineering [40]. This is to save time and energy efforts for a smooth transition of biomaterials from bench to clinic at an acceptable quality and affordable cost. Of course, further effort is required during scale-up for quality control (i.e., assessment of batch-to-batch variability and performance upgrading) [39]. The feedbacks from clinical trial phases are extremely critical for securing the quality and improving the product performances.

12.3.3 Translation and society

One of the critical issues that has been largely neglected concerning the translation of innovation is the impact of the people in society. In fact, the patients with different problems and needs are among people in society and they are the core of the healthcare system. Without patients, there will be no need for research, innovations, and regulations. As such, scientists and researchers must communicate more with society to better understand the beneficiaries of their innovations and receive feedback on the available biomedical products. It should be noted that scientists are respected and reliable members of society, so they can play a critical rule to disclose the efficacy and safety of the innovations with transparency for society. This would help patient acceptance and compliance which in turn could facilitate translation from lab to broad clinical applications [41]. In this context,

several factors are essential to consider before the implementation of innovations in the society and their acceptance such as appropriate product characterizations and labeling, cost-effectiveness and affordability, and patient consent [42].

Here, the importance of social science and information technology should be highlighted. The interaction between scientific people from different fields through an online platform and social media has resulted in good integration and tremendous advancements in the field. However, a similar interaction between scientific people and lay people or patients in society is missing. Considering the available facilities, it is now easy to address this issue through communication with public and patient organizations to discuss the issues related to opportunities, risks/benefits, and ethical concerns of new biomaterials and innovations. Therefore, the multidisciplinary nature of current advancements should call into action other disciplines such as human science, social science, law, and psychology, which would reduce the time required for translation.

12.3.4 Medico-legal and health insurance systems

There is no reliable interface between animal study and human trials that could predict the possible risks in the human model. As such, the randomized clinical trial (RCT) remains the only pathway in the modern era to support proof of efficacy, safety, and reliability for successful translation into humans. However, this translation is still largely based on the transfer of experimental data and study design and findings from animal studies despite the fact that these findings are poor predictors. In this context, since the RCT is the first attempt toward the translation of innovations, there is always uncertainty about the possible unpredicted side effects or risks that may lead to ethical–legal consequences. This uncertainty is even exaggerated in cutting-edge innovations dealing with stem cell/gene-based therapies due to the idiosyncratic nature of host responses. Today, there are some attempts to address the lack of appropriate interface between animal and human models using tissue-implant interfaces [43], tissue-on-chip or organ-on-chip systems that could better simulate the real biological responses in human [44]. However, many scientists do not believe these systems can simulate the full dynamic environmental parameters in the human organism.

Considering the lack of appropriate interface before human trial and the risk associated with phase 1 RCT, there is a critical problem related to the lack of an appropriate medico-legal framework to support the pioneers in the field for their first clinical attempt in humans [41]. Despite this fact, the field is progressing thanks to those who accept the risks to meet the challenges of finding the correct balance between risks, benefits, and innovations. The next problem is related to health insurance systems that have based their reimbursement on proof of safety/efficacy that cannot be obtained except from RCT after broad trials of new products and technologies. Similarly, the industrial sectors would request convincing reports from RCT for scale-up and mass production of new technologies. Therefore, promoting the translation of innovations into products that address the clinical needs of patients requires close cooperation between researchers and medico-legal and health insurance systems. There are some newly established agents addressing the challenges faced by innovators for translations such as the National Center for Advancing

Translational Sciences (NCATS), the NIH Centers for Accelerated Innovations (NCAI), and the NIH Research Evaluation and Commercialization Hubs (REACH).

In summary, it should be reminded that the healthcare system along with the innovations and the related regulations and policies are there to serve patients and address their clinical needs (Fig. 12.1). Close collaboration and teamwork among different agencies, organizations, and associations at both national and international levels are mandatory in order to secure a better health system for the future.

12.4 Conclusion

Despite the tremendous advancements in biomedical technologies and medicine at the laboratory phase, the translation level to address the unmet clinical needs of the patient is often disappointing. Several challenges have hindered the translation pathway such as funding, research facility, research medico-legal issues, team cooperation, confidentiality of data, data mining, conflict of interest, plagiarism, time limit, predictability, uncertainty, limited human resources, cultural/religious impact, and local/international hurdle. Despite the complexity of these challenges, the ultimate goal is to simply improve human health. Thus, the main question is how to safely translate the innovations for patient benefit considering the time limit.

Standardization of experimental protocols by researchers according to the available guidelines could be the first answer to this question. This would help to reduce the literature controversies and to facilitate data sharing which helps further advancements in the field. The next answer is in the hand of the investor, investigator, regulator, and policymaker who need to focus on the clinical problems of the patients by removing the complexities and translation barriers. It is well respected that innovations should pass through the narrow channel of safety and efficacy before clinical translation. However, the translational innovators should break the barriers by the introduction of innovations that meet urgent and unmet clinical problems in humans despite the associated risks. The rule of social media in promoting the health knowledge of society toward innovations is critical. The regulatory bodies and society should accept the possibility of failure with innovations especially when the available techniques cannot address the clinical needs. The significant cost, time, and efforts for animal studies need to be revised because of their unreliability to predict human safety and efficacy. A team-approach based on mutual trust is critical among all stakeholders of the health system. This requires a constant reevaluation of the available strategies involving the patients, academia, industries, intellectual properties, and regulatory bodies.

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SECTION II

Cellular Response to Biomaterials

Cellular response to synthetic polymers

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Abbreviations

ARG1	Arginase 1, a human protein coding gene
ALT	alanine aminotransferase
AST	aspartate aminotransferase
C3, C4	complement factor
CD11b	pan-macrophage marker
CD8 + T and CD4 + T-cells	cells of the immune system
CD68, CD14, and CD11	monocyte/macrophage markers
CFR	carbon fiber reinforced
CRBSI	catheter-related bloodstream infections
DBIL	direct bilirubin
dPTFE	high-density PTFE
ECs	endothelial cells
EGF	epidermal growth factor
ePTFE	expanded PTFE
FBGCs	foreign body giant cells
FBR	foreign body response
FDA	Food and Drug Administration
FGF	fibroblast growth factor
Fn	fibronectin
F-PLNPs	fluorophore-conjugated polystyrene nanoparticles
GBR	guided bone regeneration
GPIIb/IIIa	platelet membrane glycoproteins
HD	hemodialysis
HEC	human endothelial cells

HEP	heparin
HW	heavy weight
HXLPE	highly cross-linked polyethylene
IFN	interferon
IL, (IL)-1 β , IL-1, and IL-6	interleukin
TNF- α	proinflammatory cytokine
iNOS	nitric oxide synthase
IOLs	intraocular lenses
LW	low weight
M1	proinflammatory macrophage
M2	antiinflammatory macrophage
Mac-1	macrophage-1 antigen
MCP-1	monocyte chemoattractant protein factor-1
MGCs	multinucleated giant cells
MIP-1	macrophage inflammatory protein-1
MIP-1 α	macrophage inflammatory protein-1 alpha
MIP-1 α , MCP-1	chemokines
MSCs	mesenchymal stem cells
M Φ	macrophage
NETs	neutrophil extracellular traps
NF- κ B	nuclear factor-kappa B
PA	poly(amides)
PC	poly(carbonate)
PCL	poly(ϵ -caprolactone)
PDGF	platelet-derived growth factor
PDGF-BB	growth factor BB isomer
PE	poly(ethylene)
PEEK	poly(etheretherketone)
PEI	poly(etherimide)
PEO	poly(ethylene oxide)
PSf	poly(sulfone)
PES	poly(ethersulfone)
PET	poly(ethylene terephthalate)
PEUUR	poly(etherurethane urea)
PG	polyglactin
PGA	poly(glycolic acid)
PGE2	prostaglandin E2
PHB	poly(hydroxybutyrate)
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PLL	poly-L-lysine
PLLA	poly-L-lactic acid
PMMA	poly(methyl methacrylate)
PP	poly(propylene)
PS	poly(styrene)
PTFE	poly(tetrafluoroethylene)
PUR	poly(urethane)
PVC	poly(vinyl chloride)
PVDF	poly(vinylidene fluoride)
PVP	poly(vinyl pyrrolidone)
RBCs	red blood cells
RGD	Arginylglycylaspartic acid peptide
RONS	reactive oxygen and nitrogen species

ROS	reactive oxygen species
TBIL	total bilirubin
TCPS	tissue culture polystyrene
TGF β	transforming growth factor beta
THR	total hip replacement
TNF- α	tumor necrosis factor-alpha
TRAP	tartrate resistant acid phosphatase
TLRs	Toll-like receptors
UHMWPE	ultrahigh molecular weight polyethylene
Ym1	chitinase-like protein
α v β 3	integrin antibody

13.1 Introduction

A great number of currently available synthetic polymers makes the selection of a suitable polymer for a particular biomedical application a difficult task. However, among all properties required for an application, the biocompatibility of the polymer with tissues and biological fluids is always the foremost consideration for all candidate materials. Some synthetic polymers are key materials in several implants such as those used for joints, sutures, bone plates, and medical devices such as blood tubes, artificial heart, pace-makers, etc. They function by replacing, restoring the injured or destroyed tissues or organs, and thereby help in providing better essence of life of the patients [1]. On the basis of laboratory experimentation and clinical investigation, the following synthetic polymers are considered “biocompatible,” but mainly inert: poly(ethylene) (PE), poly(propylene) (PP), poly(tetrafluoroethylene) (PTFE), poly(urethane) (PUR), poly(vinyl chloride) (PVC), poly(amides) (PA), poly(methyl methacrylate) (PMMA), poly(acetal), poly(carbonate) (PC), poly(ethylene terephthalate) (PET), poly(etheretherketone) (PEEK), poly(sulfone) (PSU), etc. These polymers are also considered “biostable” in the body and have found wide applications in the medical field. If biodegradation of implants is desired, synthetic biocompatible and biodegradable polymers can be used. These polymers include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ϵ -caprolactone) (PCL), poly(hydroxybutyrate) (PHB), and a few other polymers.

Several classes of polymers interact with *bone tissue in applications* where they are required as artifacts to be put in direct apposition with bone or as eventual wear products of artifacts implanted in skeletal structures. Highlighting the morphological response at the interface between bone and various kinds of polymers, a common general pattern can be recognized that can be described as a “confinement reaction,” where bone tissue grows, matures, and remodels around the polymeric implant. Experiments with unloaded implants show that in the absence of inflammatory foreign body reactions to wear debris, polymers such as PE, PLA, poly(etherimide) (PEI), etc., comply with the physiologic turn-over and remodeling of surrounding bone, leading to the morphological picture of a bony rim that encloses the implant. When long-term implantation is studied, bone tissue shows the characteristic of corticalization, which means that, is likely to create a new outer border in its structure [2,3].

The cellular response of the host is dependent on medical applications and of course many other factors (highlighted in the next paragraph). In some applications the problems

related to biocompatibility and cellular response are less important. The following items are listed as the biomedical applications of the main synthetic polymers discussed in this chapter:

1. PE—*for making bone related implants, bottles used in pharmaceutical industry, pouch, containers having flexibility.*
2. PP—*for making syringes which can be disposed, oxygenator membranes used for providing oxygen to blood suture material and surgical meshes and artificial vascular grafts.*
3. PVC—*for making bag containing blood and solutions, packaging used for surgical devices, blood purification devices, catheters.*
4. PMMA—*for making pumps used to pump blood and reservoirs, membrane used for blood purification, eye related implants, ocular lenses, bone cements.*
5. Polystyrene (PS)—*for making filter devices and flasks used for tissue culture.*
6. PTFE—*for making catheter and artificial vascular grafts, meshes for hernia repair.*
7. PUR and polyamide (nylon)—*for making films used for packaging, sutures and molds, vascular prostheses.*
8. PET—*for making implantable sutures and heart valves.*
9. Poly(ethersulfone) (PES)—*catheters and lumen tubings.*
10. PEI—*skin staplers used in surgery, sterilization application.*
11. PEEK—*dentistry applications and products.*
12. PLA, poly(lactic-co-glycolic acid) (PLGA), polyanhydrides—*tissue engineering (bone scaffolds), wound management (surgical sutures, healing dental extraction wounds, and preventing postoperative adhesions), drug delivery, and orthopedic devices.*
13. PC—*hemodialysis (HD), cardiac surgery products (blood oxygenators, blood reservoirs, and blood filters), valves connectors, and transparent glassy medical instruments (trocars).*

Their biocompatibility is very important because a medical device (or component materials) should be harmless to patients. They should not be toxic or injurious and not cause an immunological response. When they are combined with other materials in a medical device, those may interact with each other which can make the medical device incompatible for use in humans. Therefore there is a need for careful testing of the components and of the entire medical device by *in vitro* and *in vivo* tests. Extractable compounds and agents and also particular monomer units, catalysts, and additives as plasticizer or other materials may produce toxicity, affect biocompatibility, and can prove to be toxic after degeneration. Some complications may appear frequently (Table 13.1).

US Food and Drug Administration (FDA) or EU approval for biocompatibility is considered important criteria. The main features to be accomplished by synthetic materials used in medical applications are: carcinogenicity (cancer causing ability), immunogenicity, teratogenicity, and toxicity (incapable of promoting any inflammatory or toxic response) should be absent; complete metabolization of medical implant is necessary after performing its function in the body, high mechanical properties, and corrosion resistance.

The typical tissue response following the implantation injury of the biocompatible materials can be divided into a cascade of events [5]. Immediately after implantation, proteins are nonspecifically adsorbed on the implant surfaces, leading to recruitment and activation of various immune cells. Subsequent release of cytokines leads to the propagation of an inflammatory process. In some circumstances, macrophages, the key players in this

TABLE 13.1 Some *in vivo* complications induced by medical devices [4].

Heart valve prostheses	Vascular grafts/stents	Cardiac assist replacement devices	Orthopedic devices	Dental implants
Thrombosis	Thrombosis	Thrombosis	Corrosion	Adverse FBR
Embolism	Embolism	Embolism	Fatigue	Fatigue
Hemorrhage	Infection	Extraluminal infection	Infection	Infection
Infective endocarditis	Disintegration and degradation	Hemolysis	Interface separation	Interface separation
Cloth wear	Proliferative restenosis	Calcification	Loosening	Loss of mechanical transfer force
Hemolytic anemia	Strut-related infection		Pain	FBR
Cups calcification	FBR		Particulate formation	Corrosion
			Surface wear	Particulate formation
				Wear

FBR, Foreign body response.

process, fuse together to form characteristic foreign body giant cells (FBGCs), which attempt to ingest the implant [6]. Finally, the implant is encapsulated in a fibrous, a vascular capsule that separates the implant from the surrounding tissue [7].

A range of signaling network of growth factors, including epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor, transforming growth factor beta (TGF β), and platelet-derived growth factor (PDGF), control adhesion, migration, proliferation [8], and differentiation of fibroblasts, keratinocytes, and endothelial cells (ECs) during injury should be followed [9].

13.2 Cellular response to synthetic nondegradable polymers

13.2.1 Poly(ethylene), poly(methyl methacrylate), and poly(tetrafluoroethylene) in bone regeneration

The success of polymers in medicine can be exemplified by the applications of ultrahigh molecular weight PE (UHMWPE), highly cross-linked PE (HXLPE), vitamin E enriched PE, and PMMA in total hip replacement (THR). These THR consisted of a stainless steel, one piece femoral component a socket made of HDPE and the implants were fixed in place with a grouting material, acrylic cement, or PMMA. The biological performance of implant materials can be evaluated by *in vitro* tests using simulated body fluid or cell cultures (as human osteoblast cell primary cultures), or by *in vivo* assessments. The response of the body to an implant is to produce a fibrous capsule, remodeling with time. If the

material is toxic, the fibrous capsule will increase in thickness; if the material is inert, the fibrous capsule has a constant thickness.

Due to high frictional torque during use, the wear particles are generated, and an aggressive chronic inflammatory and foreign body response (FBR) leading to synovitis, periprosthetic osteolysis, and loosening. Particulate debris from cemented implants with PE, generally consist of fragments of bone cement, shards, submicron PE, and more rarely, metal particulates. Periprosthetic osteolysis can be seen in areas where the biological reaction to fractured cement has initiated a chronic inflammatory reaction, also known as “cement disease.” The interface of loose cemented implants is laden with macrophages, scattered lymphocytes, and other cells in a fibrous tissue stroma. A detailed description of the role of wear particulate in biological response of such implants was presented by some research groups [4,10,11].

PE wear particles migrate within the entire “the effective joint space” (periprosthetic bed) and interact with local cells (resident phagocytic macrophages, osteoblasts, and osteoclasts), causing a cascade of proinflammatory responses or immune response with the macrophages as the key cells. Activation of macrophages occurs either by phagocytosis or contact of cell with different membrane receptors [Toll-like receptors (TLRs), CD11b, CD14]. TLRs act primarily through an adapter protein called myeloid differentiation primary response gene 88 (MyD88) to induce activation nuclear factor-kappa B and other signaling pathways [mitogen-activated protein kinase, interferon (IFN) regulatory factor 3]. TLR2 and TLR4 were highly expressed in PE-induced osteolysis. The inflammatory cascade leads to the release of various proinflammatory cytokines [interleukin (IL)-1, IL-6, TNF- α], growth factors (macrophage colony-stimulating factor-1), and chemokines [macrophage inflammatory protein-1 alpha (MIP-1 α), monocyte chemoattractant protein factor-1 (MCP-1)]. The cytokines contribute to the systemic chemotaxis of macrophages in the presence of UHMWPE particles. The chemokine receptor type 1/MIP-1 α ligand/receptor axis facilitates systemic recruitment of mesenchymal stem cells (MSCs) in the presence of UHMWPE particles. Depending upon the local environment, macrophages can be polarized to M1 (proinflammatory) and M2 (antiinflammatory) phenotypes. M1 macrophages, producers of primarily proinflammatory mediators including TNF- α , IL-1, and IL-6, express inducible nitric oxide synthase (iNOS), whereas M2 macrophages produce primarily antiinflammatory mediators including IL-4, IL-10, and IL-13, and express mammalian chitinase-like protein Ym1, Arginase 1, CD163, and chitotriosidase. Interaction with the local cells (resident phagocytic macrophages, producers of primarily proinflammatory mediators including TNF- α , IL-1, and IL-6) express iNOS, whereas M2 macrophages produce primarily antiinflammatory mediators including IL-4, IL-10, and IL-13, and express mammalian chitinase Ym1, Arginase 1, CD163, and chitotriosidase.

Therefore, by modulating the cytokine microenvironment, it is possible to decrease local inflammation to mitigate periprosthetic osteolysis. Fig. 13.1 summarizes the effect of PE particles (Fig. 13.1A) and of PMMA particles (Fig. 13.1B).

The effect of *bulk* PMMA depends on the surrounding tissue: the fibrous tissue between bone and cement underwent a metaplasia into fibrous cartilage with ossification, excellent osseointegration, and no intervening fibrous tissue around cemented components. Localized areas of osteolysis occurred due to microfractures within the bulk PMMA, that release cement particles, resulting in a localized foreign body response. Large foreign

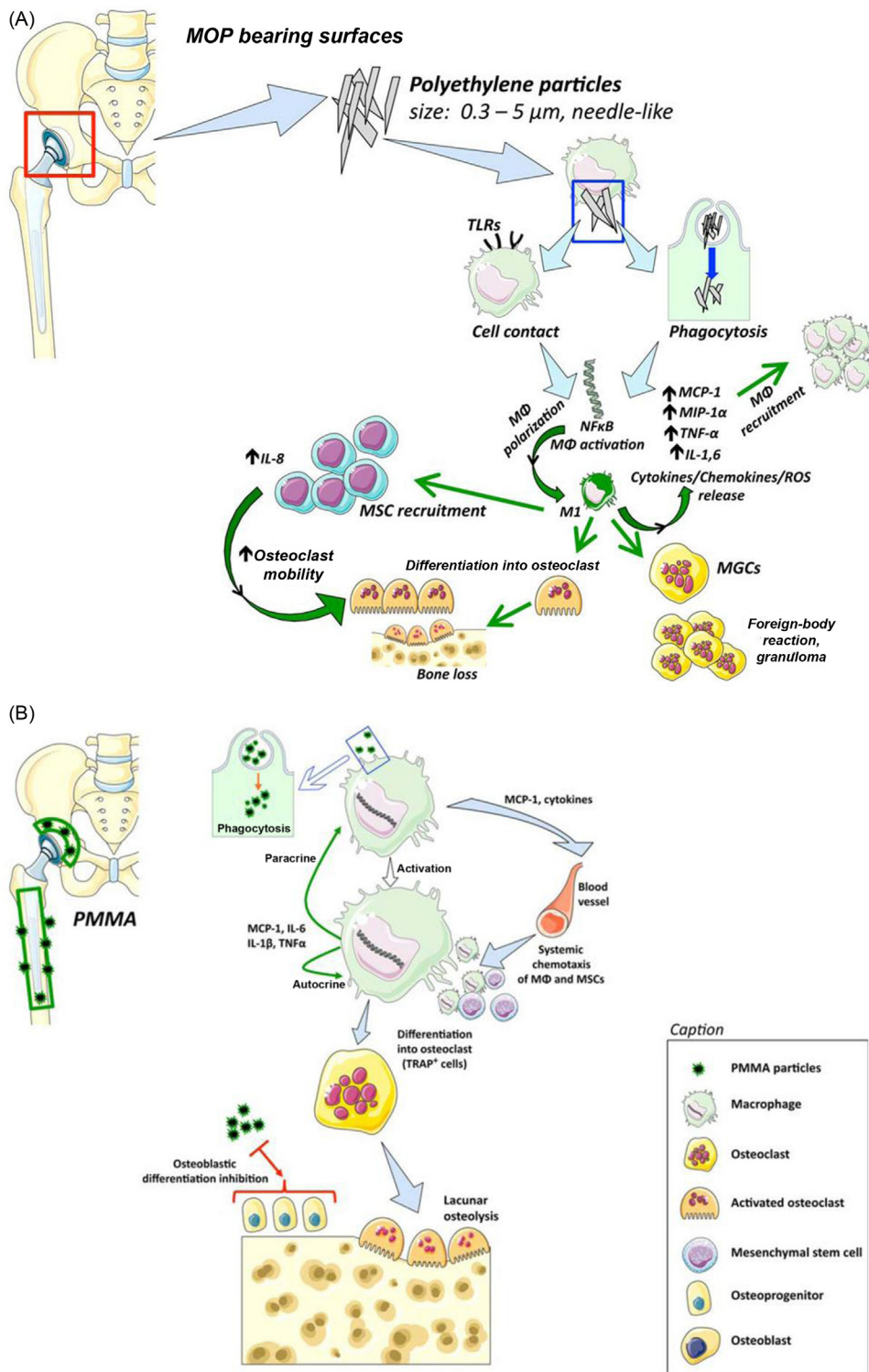


FIGURE 13.1 (A) The biological reaction to PE and PMMA. After phagocytosis or cell contact, PE particles activate nuclear transcription factors and the inflammasome. Subsequent cytokine and chemokine release occurs,

leading to systemic recruitment of macrophages. The inflammatory microenvironment polarizes M0 macrophages to proinflammatory M1 macrophages. Macrophages can differentiate into osteoclasts. MSCs increase their secretion of IL-8. Ultimately, the accumulation of osteoclasts leads to osteolysis. The fusion of macrophages leads to MGCs. (B) The biological reaction to PMMA. After phagocytosis of PMMA particles, macrophages become activated and secrete proinflammatory cytokines. The response is both local through an autocrine mechanism perpetuating macrophage activation, and systemic with recruitment of macrophages to the site of inflammation. Macrophages can differentiate into osteoclasts leading to osteolysis [4,10]. *IL*, Interleukin; *MCP-1*, monocyte chemoattractant protein factor-1; *MGCs*, multinucleated giant cells; *MIP-1 α* , macrophage inflammatory protein-1 alpha; *MSCs*, mesenchymal stem cells; *M Φ* , macrophage; *NF- κ B*, nuclear factor-kappa B; *PE*, poly(ethylene); *PMMA*, poly(methyl methacrylate); *ROS*, reactive oxygen species; *TLRs*, Toll-like receptors; *TNF- α* , tumor necrosis factor-alpha; *TRAP*, tartrate resistant acid phosphatase.

body granulomas and giant cells with PMMA debris inside the cells were found. The response mainly consisted in the production of a thin fibrous membrane with occasional giant cells, lymphocytes, and histiocytes. During polymerization, PMMA releases free radicals that have been shown to be cytotoxic for osteoblastic cells.

The PMMA particles influence the chronic inflammatory reaction to bone cement breakdown products and lacunar osteolysis. The osteolytic potential of PMMA has been associated with increased production of proinflammatory cytokines, increased release of MCP-1, and IL-6. PMMA particles increased the levels of IL-1 β and TNF- α .

There are many factors that affect cellular interactions and many cellular processes (cellular differentiation, DNA/RNA transcription, cell metabolism, and protein production), such as surface wettability (hydrophilicity/hydrophobicity or surface free energy), chemistry, charge, roughness, and rigidity of dynamics of polymeric materials. The surface properties play an important role for the morphology of adhesion, growth, and differentiation of cells.

Kato et al. found that cells interact with material surfaces through expressing special genes, such as genes encoding IL-1 β and heat-shock protein [12,13], protooncogene, and tumor suppressor gene, when the material surface has a particular wettability.

Kim et al. studied by MTT assay, spreading and adhesion, the growth and proliferation rate of NIH/3T3 fibroblast cells on LDPE surfaces obtained by plasma treatment. It has been established that the cell adhesion, the proliferation rate of cells was higher on film surface with a water contact angle of 50–60 degrees than with other water contact angle [14,15] while *c-fos* mRNA and cellular mRNA such as *c-myc*, *c-fos*, and *p53* mRNA in cells showed maximum expression on these films. Therefore the polymer surfaces for which a water contact angle of 50–60 degrees was obtained, and are suitable for cell adhesion and growth, as well as for biological responses for adhesion, proliferation, and tumor suppression of fibroblasts—Fig. 13.2 [16]. Cells did not become carcinogenic and maintained tumor suppressor function on the plasma-treated film.

13.2.1.1 Inflammatory changes preceding osteolysis

Following a large total joint arthroplasty, a newly formed joint capsule or pseudosynovial membrane is typically formed around the implant within the joint space. The predominant cells found in the intermediate fibrous membrane and other periprosthetic tissues include fibroblasts, histiocytes, infiltrated peripheral blood monocytes, and multinucleated giant cells (MGCs) (Fig. 13.3). The UHMWPE particles <2 μ m, are ingested and found within

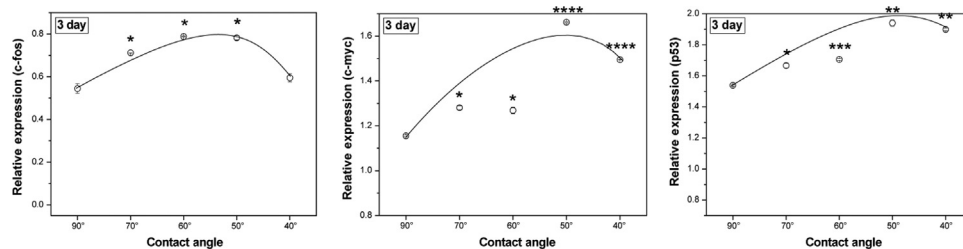


FIGURE 13.2 Expression of various cells adhered on plasma-treated surface on day 3. Asterisk denotes significant differences of two figures as determined by Student's *t*-test; for contact angle of 50-60 degrees; * $P < 0.005$; ** $P < 0.0005$; *** $P < 0.0001$; **** versus 90 degrees [16].

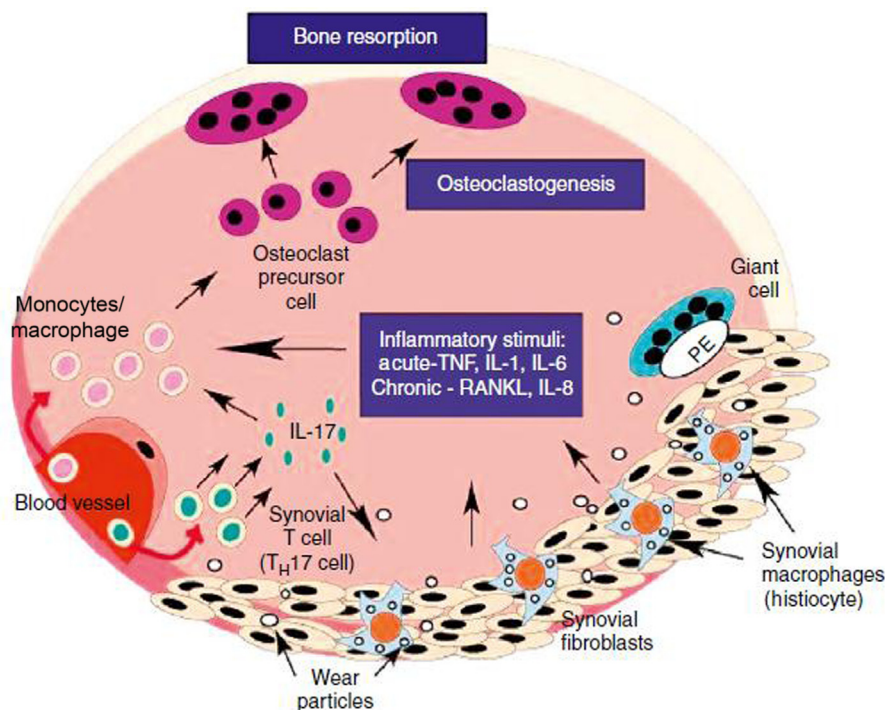


FIGURE 13.3 Diagram of inflammatory changes preceding osteolysis. The infiltration of immune cells, including monocytes and T-cells, and activation of resident synovial fibroblasts and histiocytes in response to UHMWPE wear debris leads to the production of chemokines, cytokines, and growth factors. Giant cells form in response to larger wear debris. Monocyte/macrophages differentiate into osteoclasts, which are the cells responsible for bone resorption [17]. UHMWPE, Ultrahigh molecular weight polyethylene.

phagosomes of the histiocytes and macrophages referred to as phagocytosis or frustrated phagocytosis. The fusion of macrophages to form giant cells occurs in an attempt to ingest the UHMWPE particles, usually $>10 \mu\text{m}$, because $5 \mu\text{m}$ particles have been observed within these MGCs. Activation of cells marks the beginning of a chronic inflammatory reaction

initiated by the accumulation of UHMWPE wear debris and the inability of the phagocytic cells to degrade the ingested or uningested wear debris. During this process, the cells release protein degrading enzymes and destructive reactive oxygen and nitrogen species (RONS). The innate immune response induced by wear particles is a chronic inflammatory and non-specific foreign body reaction. Activated mast cells as immune cells found in mucosal and connective tissue in relatively low numbers dramatically increase, and the release of enzymes and other mediators which stimulate fibroblast proliferation, vascularization, and inflammation lead to the production and release of cytokines, chemokines, and growth factors. The acute response proinflammatory cytokines and chemokines produced by activated fibroblasts, macrophages, and mast cells include IL-1 α , IL-1 β , IL-6, and TNF- α . In most inflammatory reactions in acute phase, IL-6, IL-8, and MCP-1 are detected in these tissues and also four types of hypersensitivity immune response.

Morphologic changes found in tissue and bone surrounding the implant include tissue fibrosis, necrosis, fibrocartilage formation, heterotopic ossification, and last, but certainly not least, osteolysis of the surrounding bone with the contribution of both cement and UHMWPE wear debris after a year of implantation. After 2.5 years, the number of histiocytes contained large amounts of phagocytosed wear debris with degenerative changes in chromatin structure and the disintegration of cell borders and within the fibrous membrane dramatically increased concurrently with the cement wear debris. A positive correlation with implant duration was found only for necrosis and the increasing thickness of the fibrous tissue over time correlated with the increasing number of histiocytes and amount of wear debris. Morphologically, necrotic cells appear swollen and show signs of nuclear and cytoplasmic degeneration [18].

13.2.1.2 Osteolysis

UHMWPE wear debris-mediated osteolysis is widely recognized as one of the most serious challenges in hip arthroplasty [19,20].

Periprosthetic osteolysis is the process by which biological or mechanical forces initiate a local immune response in periprosthetic tissue that eventually results in implant loosening and failure [21]. This could be due to a number of factors such as infection, wear-induced osteolysis which results in a unique immune response dependent on both particulate dose and size [22,23]. Evaluation of clinical data showed that UHMWPE particle quantities in the order of 10 billion particles per gram weight of tissue, suggests the existence of a threshold for the infiltration and activation of monocyte/macrophages and the onset of osteolysis. Soluble products released by activated macrophages and fibroblasts in the periprosthetic tissue control the activity, proliferation, and differentiation of osteoclasts, bone-degrading cells, and osteoblasts, bone-forming cells (Fig. 13.3). Osteoclast (monocyte/macrophage precursors) differentiation, fusion to form multinucleated cells, and activation result from the binding of receptor activator of nuclear factor- κ B ligand (RANKL) to its receptor RANK. IL-6 is produced by monocyte/macrophages and fibroblasts and enhances the recruitment of osteoclast precursors, promoting osteoclast formation (osteoclastogenesis). Bone resorption by osteoclasts includes degradation of the organic and inorganic parts of bone after differentiation and fusion by creating a sealing zone between the bone and the osteoclast, into which protons, degradative enzymes (e.g., cathepsin K),

and RONS are released. Proton pumps produce an acidic pH, causing hydroxyapatite, the mineral making up the bone, to be dissolved.

UHMWPE wear particulates generally range from 0.1 to 1.0 μm (average 0.5 μm) in length when recovered from the hip, but average $\sim 280 \mu\text{m}$ in length when recovered from the knee. UHMWPE particulates of about 0.24 μm in length elicited the greatest rate of bone resorption and cytokine [tumor necrosis factor (TNF)- α , IL-1 β , IL-6, and prostaglandin E2] production. Large particles are trapped in dense, collagen-rich joint connective tissue, while smaller than 1 μm particles are able to move more freely. Particles $>50 \mu\text{m}$ were identified in abdominal lymph nodes, spleen, and liver. Lymphatic transport through perivascular lymph channels, as free or phagocytosed particles within macrophages, is the most probable route for wear debris distribution [17,24]. The overall pathophysiologic response to UHMWPE wear debris is a complex process, involving a number of cell types and progressive local and systemic changes with increasing implantation times. Potential factors involved in individual-specific responses include genetic polymorphisms in matrix metalloproteinase 1, IL-6, TNF- α , and the vitamin D receptor. Three general cellular responses have been described, depending on particulate size. Particles 150 nm are pinocytosed, particles 150 nm to 10 μm are phagocytosed, and particles 420 μm induce multinucleated giant cell formation, with some overlap. Smaller particles ($<1 \mu\text{m}$) are phagocytosed by macrophages, whereas larger sized particles ($>10 \mu\text{m}$) are generally surrounded by many macrophages and FBGCs (frustrated phagocytosis). Submicron-sized wear particles are phagocytosed leading to increased production of cytokines by macrophages. Increased TNF- α , IL-1, and IL-6 levels were found in the interfacial tissue from hip replacements with osteolysis. The activated macrophages secrete cytokines, chemokines, and other factors that recruit more macrophages to the local area, induce osteoclast maturation, and eventually cause periprosthetic osteolysis.

Numerous cell types and inflammatory mediators are implicated (Table 13.2), although cells of monocytic lineage dominate in the pathogenesis of osteolysis.

Injury to the tissue or organ initiates an acute inflammatory reaction in addition to the specific reaction to the biomaterial itself. Several factors are responsible by the extent of injury, *protein absorption, coagulation, complement activation, and migration of leukocytes* to the area.

1. *Proteins* from the blood and interstitial fluid immediately adsorb to the material surface prior to interact with host cells. The composition, size, shape, surface roughness, surface chemistry, hydrophobicity, and surface charge influence the types, concentrations, and conformations of the adsorbed proteins on the surface. These proteins subsequently determine the adhesion and survival of cells, especially polymorphonuclear neutrophils, monocytes, macrophages, and FBGCs. The presence of adsorbed protein such as albumin, fibrinogen, complement, fibronectin (Fn), vitronectin, and γ -globulin determine and modulate cell adhesion and intercellular interactions on the implant surface, thereby influencing the subsequent wound healing response. This protein adsorption phenomenon is the equivalent of the Vroman effect for biomaterials in contact with blood.
2. *Coagulation* is initiated by factor XII and tissue factor resulting in the activation of prothrombin to thrombin. The minute amount of thrombin activates platelets to release mediators of the coagulation system and exposes negatively charged phospholipids,

TABLE 13.2 Essential cells and inflammatory mediators (molecular effector) involved in the biological response (e.g., in osteolysis) to orthopedic implants for joint replacement of ultrahigh molecular weight polyethylene (UHMWPE) and poly(methyl methacrylate) [4,10,21,25].

Cells or inflammatory mediator type	Role/function
<i>Cytokines and chemokines</i>	
MCP-1	Immediate early stress-response factor. Important in systemic migration of MΦ to local site. Produced by monocytes and activated NK cells, fibroblasts, and bone-marrow derived primary osteoblasts
MIP-1α	MIP-1α enhances the release of IL-1 and IL-6 affecting neighboring cells in a paracrine manner. Produced by activated MΦ and T lymphocytes.
IL-1α and IL-1β	IL-1 activates MΦ, neutrophils, and endothelial cells; stimulates fibroblasts and osteoclasts, and induces prostaglandin E2 and collagenase synthesis. IL-1α and IL-1β are produced by two distinct genes. Secreted by many cell types including macrophages
IL-1	Increases RANKL expression; inhibits osteoclast apoptosis; mediates TNF-α-induced RANKL expression; enhances osteoclastogenesis in presence of RANKL60; capable to activate MAPK and NF-κB48
IL-6	Secreted by osteoblasts in response to wear particle, IL-1β, and TNF-α stimulation; secretion induced by NF-κB activation; released by stimulated macrophages and associated with increased osteolysis; stimulates RANKL expression on osteoblast cell surface in inflammatory state
IL-6	Activates T and B cells and induces B cells to differentiate and secrete immunoglobulins. Secreted by macrophages, T-cells, fibroblasts and other cell types
TNF-α	Stimulates fibroblasts and granulocytes; some of the effects are similar to IL-1. Secreted by activated lymphocytes, monocytes, MΦ and other cells. Increases RANKL expression and RANKL-induced osteoclastogenesis; activates osteoclasts with similar potency of RANKL; inhibits osteoclast apoptosis by Akt and ERK phosphorylation; strongly suppresses procollagen 1 expression; increases IL-1 and IL-1R type I expression in vitro; enhances macrophage-attractant chemokine production
PDGF-α	Increases class II antigen expression in macrophages, stimulates osteoclasts to resorb bone, induces collagenase and prostaglandin production, and is chemotactic for fibroblasts, monocytes and neutrophils. Secreted by MΦ, platelets, endothelial cells and fibroblasts
TGF-β	Stimulates fibroblast growth, extracellular matrix formation and suppresses T- and B-cell proliferation. TGF-β also stimulates osteoblast and inhibits osteoclast function. Secreted by T-cells, activated MΦ, and other cell types
<i>Inflammatory mediators (molecular effector)</i>	
Monocyte/macrophage	Dominant in osteolysis: are present in periprosthetic tissue and pseudosynovial membrane, being correlated with bone resorption. Secrete MMPs, TNF-α, IL-1β, IL-6, and eicosanoids after phagocytosis of wear debris; their migration contributes to local infiltration, osteoclast differentiation, and bone destruction; increased peripheral CD14 ⁺ CD16 ⁺ phenotype frequency; correlated with increased production of TNF-α and IL-1β52

(Continued)

TABLE 13.2 (Continued)

Cells or inflammatory mediator type	Role/function
DCs	Infiltrate and surround UHMWPE particles, participating in phagocytosis and MGC formation; produce proinflammatory cytokines IL-1, IL-6, IL-12, TNF- α , and IFN- γ ; contribute to osteoclastogenesis and activation
Osteoblast	Able to phagocytose wear particles and alter cellular signaling; decrease OPG secretion; express RANKL104 and M-CSF105 upon TNF- α and IL-1 β stimulation in vitro; dose-dependent decrease in proliferation, differentiation, and mineralization; decreased expression of procollagen α 1 mRNA and synthesis of collagen I in vitro by phagocytic-dependent and –independent mechanisms when challenged; increased expression of IL-6101 and IL-8103
Fibroblast	Present in pseudosynovial periprosthetic membrane; expresses RANKL and can induce osteoclast differentiation in pseudosynovial membrane; activated by TNF- α and IL-1 β and osteoarthritis108 models; possess MMP2 mRNA alongside macrophages and MGCs expressing MMPs 2 and 14 protein but only MMP14 mRNA109
RANKL	Inhibits osteoclast apoptosis
NALP3 inflammasome	Activated by cathepsin and ROS, in conjunction with other intracellular danger signals (ATP, K ⁺ , urate, etc.); complexes with PYCARD, leading to caspase-1 recruitment and activation
Caspase-1	Regulator of inflammation and cell survival and differentiation; cleaves pro-IL-1 β and pro-IL-18 into active forms, allowing for their secretion; Increased levels, along with IL-1 β and IL-18, after in vitro treatment of DCs with UHMWPE; this response diminished with cathepsin inhibition
TLR	Nonspecific and self-propagating immune response, indicative of the innate immune system; Activated by DAMPs released in tissue damage; activation by alkane polymers enhanced by polymer oxidative damage up to 140-fold; DC, monocyte, macrophage, and osteoclast activation, with upregulation of MHC-II, B7-1, B7-2, CD40, IL-1, IL-6, IL-10, IL-12, TNF- α , and IFN- γ , as well as TLR1 and 2; TLRs 2, 4, 5, and 9 observed in monocytes/macrophages of osteolytic tissue in vitro, with TLR2 and 5 response dominant; TLR9 characterized as strongest promoter of phagocytosis in a bacterial model
Complement	PE activates alternative complement pathway, likely through Factor B; complement factors adsorbed to PE particles after activation; complement activation enhances vascular permeability, chemotaxis, and phagocytosis; C3 demonstrates ability to recruit osteoclasts, and activate NF- κ B40
MMP	MMPs 1, 9, 10, 12, and 13 highly elevated in AL periprosthetic tissue, in addition to lesser elevation of others; MMPs 1, 2, 3, and 9 identified in macrophages, fibroblasts, and endothelial cells of AL periprosthetic tissue; Combined action capable of degrading almost all elements of periprosthetic extracellular matrix
MAPK	Activated by TNF- α , IL-1, and IL-18; signaling leads to IL-1 and other cytokine expression, as well as NF- κ B activation

DAMP, Damage-associated molecular pattern; DC, dendritic cell; IFN- γ , interferon- γ ; IL-1, interleukin-1; M-CSF, macrophage colony-stimulating factor; MGC, multinucleated giant cell; MHC-II, major histocompatibility complex class II; MMP, matrix metalloproteinase; NF- κ B, nuclear factor-kappa B; OPG, osteoprotegerin; PE, polyethylene; RANKL, receptor activator of nuclear factor- κ B ligand; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF- α , tumor necrosis factor- α .

thus providing the necessary catalytic surface for the coagulation cascade. Integrin-bonding domains on phagocytes are activated by fibrinogen/fibrin adhered to biomaterials, further initiating the inflammatory response and blood clot formation.

3. *Complement activation* is associated with the adsorbed protein layer. Attached IgG binds C1q forming a C1 assembly. The initiation of classical C3 convertase generates C3b that binds to the biomaterial protein layer to form more C3 convertase. Thus a complement cascade is initiated, high amounts of C3a and C5a are generated at the implantation site. The anaphylatoxins contribute to the onset of inflammatory responses at the implantation site through their multitude of effector functions as mast cell degranulation, increasing vascular permeability, attracting and activating granulocytes and monocytes, and inducing the release of granulocyte-reactive oxygen species (ROS).
4. *Inflammatory cells.* Leukocytes migrate from the blood vessels to the perivascular tissues at the implantation site and they accumulate through margination, adhesion, emigration, phagocytosis processes, and extracellular release of leukocyte contents. Protein ligands of integrins as major adhesion receptors of leukocytes are fibrinogen, factor X, iC3b, Fn, and vitronectin. IL-4 and IL-13 are released from mast cells during the degranulation process and determine the extent of the subsequent foreign body reaction. Polymorphonuclear leukocytes represent a source of immunoregulatory signals which are synthesized upon activation. IL-8 chemokine polymorphonuclear leukocyte is the most prominent. Activated polymorphonuclear leukocytes secrete monocyte chemotactic protein-1 (MCP-1 or CCL2) and MIP-1. Polymorphonuclear leukocytes undergo apoptosis after they fulfill their roles as phagocytes and are subsequently engulfed by macrophages. Within the first 2 days after biomaterial implantation, polymorphonuclear leukocytes typically disappear from surgical sites.

The innate immune response is activated pattern recognition receptors such as TLRs (TLR2, TLR4, TLR5, and TLR9 expressions) in the presence of chemical sequences of bacterial-derived pathogen-associated molecular patterns PAMPs and host-derived damage-associated molecular patterns adhering to the surface of implanted devices or being associated with the generated wear particles. PMMA particles without PAMP can activate macrophages and induce osteolysis in a TLR pathway-dependent manner.

More clinical trials are needed to understand cellular response and the role of the cells and of the inflammatory mediators.

The hip, knee, shoulder, or spine, as UHMWPE components are exposed during manufacture, sterilization, and shelf aging are bathed in body fluids containing dissolved oxygen and reactive free-radical oxygen species potential initiators, for *in vivo* oxidation. *In vivo* oxidation of PE is mediated by biological molecules, such as lipids (FTIR evidence—carbonyl region ($1710\text{--}1740\text{ cm}^{-1}$) or 1718 cm^{-1} associated with ketone band or 1600 and 1670 cm^{-1} hydroperoxide content) are complicated by the presence of biological contamination, mainly in the form of lipids and proteins. On the other hand, UHMWPE is not a static material but is continually undergoing dynamic changes *in vivo*. The increasing density, crystallinity differential scanning calorimetry, and decreasing molecular weight with implantation time was observed. Density changes in UHMWPE could be correlated with changes in mechanical behavior (e.g., higher elastic modulus, higher stresses in implants and greater surface damage) [26,27]. Using gas chromatography–mass spectrometry, squalene, cholesterol, and

cholesteryl derivative were found as the main diffused products [28]. At elevated levels of oxidation, the ultimate strength and ductility of UHMWPE become compromised and fatigue failure [29,30]. An oxidation index is defined by (ASTM) F2102: low oxidation $OI < 1$ ($1 \leq OI \leq 3$; $OI > 3$ critical oxidation). The oxygen dissolved in the joint fluid, rather than peroxide radicals, is responsible for in vivo oxidation.

The osteolysis and aseptic loosening of the prosthesis leads to develop new materials for modern total hip arthroplasty (about 6921 articles) [31].

Oxidation decreases resistance of the biomaterial increasing wear [32]. Stabilization of conventional UHMWPE is necessary. Biologic activity of the wear debris was reduced and osteolysis has been dramatically decreased by manufacture of highly cross-linked PE [33,34].

High cross-linked UHMWPE (XLPE) is aimed at improving UHMWPE in both cemented and uncemented implants to decrease PE wear, to improve wear resistance while maintaining mechanical properties and eliminating the oxidation process [35]. High cross linking density is obtained using gamma irradiation or electron beams at a dose between 50 and 100 kGy, to increase wear resistance. Heat treatment is aimed for eliminating free radicals that appear after crosslinking; this thermal treatment applies temperature above (remelting) or below (annealing) the melting transition temperature of the polymer (137°C).

Antioxidant doped PE improves oxidation resistance without compromising mechanical properties through thermal treatments. XLPE is stabilized by the addition of antioxidants like vitamin E, to prevent oxidation of free radicals with the intention of increased wear resistance [36–38].

Poly(2-methacryloyloxyethyl phosphorylcholine), which is formed by photoinduced graft polymerization, creates a superlubricious layer that mimics articular cartilage [39]. A recent hip simulator study reported that MPC polymer grafted on the XLPE surface dramatically reduced the wear up to 70 million cycles [40].

Guided bone regeneration (GBR) is widely used in the fields of periodontology, implant dentistry, and maxillofacial surgery. Dental barrier membranes allow for the formation and maintenance of spaces, which, when filled with bone substitutes, stabilizes blood clots, and allows the migration of osteoprogenitor cells into the space intended for bone regeneration, while preventing the area from soft tissue penetration or collapse [41]. In this context, barrier membranes have to fulfill the following main criteria: separation of hard and soft tissue up to the time point of completed bone regeneration, biocompatibility, space-maintenance, cell-occlusiveness, tissue integration, and clinical manageability, amongst other different requirements [42].

Different barrier membranes are available for GBR procedures, which can mainly be divided into resorbable and nonresorbable materials. Although resorbable membranes are preferred due to the avoidance of a second surgery, clinical situations such as bone defects outside the ridge contour, multiwalled bone defects, or vertical augmentations require maintenance of the spatial barrier, which can be achieved by the application of nonresorbable materials such as PTFE membranes [43].

Commercially available nonresorbable barrier membranes are most often made of PTFE. PTFE has been shown to be biocompatible, and it maintains its integrity during and after implantation. In this domain, both high-density PTFE (dPTFE) and semipermeable expanded PTFE (ePTFE) membranes are available, both providing different advantages [44]. While

semipermeable PTFE membranes may support a transmembraneous transport of nutrients, dense PTFE membranes have shown to act as an efficient barrier against bacterial and cellular penetration in different clinical indications, due to its small pore size [45]. Some PTFE membranes are even combined with structural elements such as titanium (Ti) [46].

Korzinskas et al. studied the possibility of using a dPTFE nonresorbable polymer as a barrier membrane designed for being applied to support bone healing within the context of GBR [47]. It was observed that dPTFE membrane induced a tissue response, including inflammatory cell types such as macrophages and granulocytes, up to day 30 postimplantation. Detection of both macrophage subtypes showed that more CD206-positive M1 macrophages were present at day 10 after implantation, compared to macrophages expressing the M2 phenotype; this tissue reaction pattern was found to be comparable to a control collagen membrane. The analysis showed a decrease of proinflammation reflected by the significant reduction of M1 macrophages at 30 days in both groups. Although no differences between the numbers of CD206-positive cells within the implantation beds of both biomaterials have been measured, the decrease of M1 macrophages was more pronounced in the case of the dPTFE membrane, as expressed by the higher significance level, as in case of the collagen membrane. Taken together, the significantly higher proinflammatory tissue response at day 10 after implantation was reduced at day 30 to a comparable level of M1 and M2 macrophages, even in the case of the dPTFE membrane.

Human osteoblasts were grown on the inside and outside surfaces of a clinically available ePTFE graft material, using a short-term primary cell culture system to examine the osteoblast morphology and mitogenic response [48]. The vascular graft PTFE was chosen for being used in interposition arthroplasty for the carpometacarpal joint. It was observed that patent grafts remained essentially free from fibrous tissue penetration after implantation from 1 to 60 months [49]. Other types of Gortex grafts demonstrated a range of cellular ingrowth from 24 hours to 2 weeks [49]. In the study of Walsh et al. [48], the cultured human osteoblasts did not appear to grow or spread on either surface of PTFE or respond to a mitogenic stimulus of 20 ng/mL of growth factor BB isomer (PDGF-BB). The percentage of area covered by the osteoblasts did not increase with time on either the inside or outside surfaces of PTFE.

13.2.2 Poly(propylene), poly(tetrafluoroethylene), and poly(ethylene terephthalate) as surgical meshes

PP is a nonmetallic, synthetic, nonabsorbable material, resistant to infection, tested together with PTFE, Dacron, Orlon, PE, Mylar, and Marlex used in hernia repair. PP is lightweight, flexible, strong, easily cut, readily integrated by surrounding tissues. As monofilaments it provides large pores facilitating fibrovascular ingrowth, infection resistance, and improved compliance. PP remains the most popular material in mesh hernia repair [50]. PP possesses attributes of an ideal suture. PP (Prolene Ethicon), a synthetic nonabsorbable, less thrombogenic, and relatively inert material is used for suture. PP has been suggested for intestinal surgery [51–53]. PP had a consistently higher adhesion when compared with polyglactin (PG) 910 (a braided multifilament synthetic absorbable suture material that has been recommended for both renal and gastrointestinal surgeries). The low inflammatory cell response in

the PP group could be a reflection of the relative inertness of PP, which had earlier been reported [54]. Mesh materials must also possess the biomechanical properties (flexibility, strain values greater than 30%) and do not stretch more than the native human abdominal wall in any direction, larger pores, the tensile strengths of more than 100 N/cm of conventional heavyweight meshes (e.g., Prolene), a specific orientation as necessary to withstand the stresses placed on the abdominal wall. Most synthetic meshes, even the lightest meshes, reach a tensile strength of at least 32 N/cm and are sufficiently strong. Other mechanical properties are: elongation and orientation. The mechanical compatibility between the hernia meshes and the abdominal wall layers plays an important role in avoiding postoperative complications and recurrences. In most situations, a lightweight monofilament mesh (e.g., PP or POL mesh), with large pores and minimal surface area is preferable. In order to improve the host compatibility to mesh and improve the tensile strength of the prosthesis, many commercially available meshes today provide an absorbable or nonabsorbable coating over polyester or PP meshes [55]. A relationship was found between the higher fibroplasia and the adhesion observed in the PP group. Since fibroblasts are the precursors of collagen also involved adhesion, the higher fibroplasia may be due to the severe adhesion. Various meshes are also tested. Their pores must be more than 75 μm in order to allow infiltration by macrophages, fibroblasts, blood vessels, and collagen. Meshes with larger pores allow increased soft tissue ingrowth and are more flexible because of the avoidance of granuloma bridging as part of the foreign body reaction. Bridging individual granulomas become confluent and encapsulate the entire mesh. Reduced bridging was associated with reduced mesh contraction [56–58]. In the case of a mesh with pores $<800 \mu\text{m}$, granuloma is more likely to form. Multifilament meshes have smaller pore sizes $\leq 10 \mu\text{m}$, inhibiting rich collagenous ingrowth and immune cell surveillance. Lightweight meshes typically 33 g/cm² initiate a less pronounced foreign body reaction, a decreased inflammatory response, better tissue incorporation, increased compliance of the prosthesis, and decreased patient discomfort and pain. PP provokes a chronic inflammatory response that degrades PP via oxidation, and the mechanical properties of the mesh are altered. Mesh contraction occurs in all synthetic meshes. Therefore the choice between a lightweight and heavyweight mesh is multifactorial. Meshes are used for the clinical use of surgical meshes (used in urological and gynecological surgery) as hernia repair and in surgical meshes for vaginal prolapse surgery, abdominal wall surgery [59], in pelvic reconstructive surgery, etc. Mesh implants are composed of nonabsorbable PP, PET, ePTFE, poly(vinylidene fluoride) (PVDF), and absorbable materials, such as PLA, PGA, PHB, PCL, and polydioxanone, with different textile structures (mono or multifilament fibers with different textile design as knitted structure, warp-knitted structure, nonwoven structure, and woven structure and with isotropic, anisotropic, or combined orientation). Nylon was the first plastic material used as a suture and was later woven into mesh prosthesis for hernia repair, but it proved to be not suitable in hernia repair, because it lost strength over time due to hydrolytic digestion and it required explanation if infected [50].

Types of mesh vary substantially with regard to raw material (structure, composition of the fibers) type of weave, pore size, mechanical parameters (tensile strength, interstices characteristics, isotropy and flexibility of the material), all these helping in the selection of a mesh implant which should match the physiological conditions. The structural parameters, especially the porosity, are the most important predictors of the biocompatibility performance of synthetic meshes. Potential mesh-related complications include chronic infections, chronic

pain and mesh rupture. Meshes with large pores exhibit less inflammatory infiltrate, connective tissue and scar bridging, which allows increased soft tissue ingrowth. Based on various factors, a classification of meshes is presented in Table 13.3 [66].

Types 1 and 2 meshes are used in pelvic reconstructive surgery, types 3 and 4 meshes are instead used for prostheses, such as vascular grafts. Ideal mesh properties used for pelvic reconstructive surgery should result in minimal infection, minimal inflammatory reaction, adequate fibrosis, and avoidance of excessive fibrosis. Host response also varies on physical properties of individual mesh (pore size, weight, coatings, bacterial colonization, and biofilm production) of PP for vaginal surgery for pelvic floor reconstruction [61].

In the 20th century, multiple biomaterials were used, but were abandoned due to sub-optimal results or complications, and the most widely used remain: PP with maximum strength, polyester, and the ePTFE.

Material and filament composition of mesh is the main factor in determining cellular response (type 3 meshes), having the highest proportion of giant cells and histiocytes. Type 1 meshes and Dexon mesh had minimal giant cells or histiocytes. Type 1 monofilamentous PP (Atrium, Gynemesh, Prolene, SPARC and TVT) and type 3 (Vypro II as PP and PG, Dexon and IVS—multifilamentous) meshes demonstrated different biocompatible

TABLE 13.3 Mesh classification [60–65].

By pore size	By weight	By biomechanical stability and elasticity. Anisotropy, porosity
1. Macroporous pore size >75 μm ; the size required for infiltration of macrophages, fibroblasts, blood vessels	1. Ultralight $\leq 35 \text{ g/m}^2$	1. Large pore mesh (textile porosity of >60% or effective porosity of >0%): a. Monofilament b. Multifilament c. Mixed structure or polymer, that is, combination of absorbable with nonabsorbable, or different varieties of nonabsorbable materials
2. Microporous pore size <10 μm in at least one of their three dimensions	2. Lightweight 35–70 g/m^2	2. Small pore mesh (textile porosity of <60% and without any effective porosity). a. Mixed structure or polymer
3. Macroporous with multifilamentous or microporous components	3. Standard 70–140 g/m^2	3. Meshes with special features, for example, covered meshes or composite meshes for intraabdominal use or meshes with surface coatings
4. Submicronic pores sizes	4. Heavy $\geq 140 \text{ g/m}^2$	4. Meshes with films, for example, meshes without porosity, submicronic pore size
		5. 3D meshes, for example, preshaped or 3D devices
		6. Biological meshes: these can be further classified onto absorbable or nonabsorbable and, based on source, into synthetic or biological; further subgrouped into: a. Noncrosslinked b. Cross-linked c. Special features

3D, Three-dimensional.

properties. Inflammatory cellular response and fibrosis at the interface of mesh and host tissue was most marked with Vypro II and IVS [67]. All type 1 meshes displayed similar cellular responses despite markedly different mesh architecture [68]. Most available prosthetic meshes in use today are based on these materials. The inflammatory response and fibrous reaction in the nonabsorbable type 3 meshes combined multifilamentous PP and multifilamentous PG tested (IVS and Vypro II) was more marked than the type 1 meshes. The increased inflammatory and fibrotic response may be because of the multifilamentous PP components of these meshes. There is a higher rate of infection with multifilaments rather than monofilaments of the same material. A theoretical disadvantage of multifilaments is that their interstices are less than 10 μm and this may allow small bacteria to infiltrate and proliferate. The bacteria may then not be eliminated by macrophages and neutrophils, which may be too large to enter a 10- μm tridimensional pore. In all mesh materials: PP, Prolene; PET, Mersilene; and PP + PG, Vypro, a persisting T-cell response was observed. Colonization of the interface with macrophages showed a pronounced reduction in the PP + PG mesh group. Index of mast cells increased in the PP mesh group and indices of proliferation were highest [69].

PET, commonly called polyester, is made up of ethylene glycol and terephthalic acid. Polyester is hydrophilic in nature and hence a propensity to swell when in contact with tissue fluids. Though it induces a similar biological response as PP, polyester is known to degrade with time, especially during infections, therefore claiming for hernia repair [70]. This effect is accentuated in an infected environment [71]. The meshes are available in multiple configurations for inguinal, hiatal, and incisional hernia repair [68]. In addition, the mesh for ventral incisional hernia repair is coated with collagen, similar to PP-coated mesh to prevent adhesions and can, thus, be used for intraperitoneal repair. This mesh is chosen for hernia repair mainly to improve conformability and tissue in-growth with the abdominal wall [72]. Its biological response in terms of scar formation, side effects, and complications is similar to that of PP [73].

Covidien (an Irish-headquartered global health care products company and manufacturer of medical devices and supplies) came out with different designs of polyester meshes specially designed for laparoscopic use [74] (Table 13.4).

Another synthetic polymer often used for obtaining meshes for hernia repair is PTFE, a chemically inert synthetic fluoropolymer which has a high negative charge, therefore water and oils do not adhere to it. This material does not incorporate into human tissue and becomes encapsulated. Poor tissue incorporation increases hernia recurrence and an infected PTFE mesh must be explanted. PTFE is microporous, which allows bacteria passage but prevents macrophage passage; therefore the body cannot clear the infection [75,76]. To obtain an improved form, PTFE was ePTFE, and it became a uniform, fibrous, and microporous structure with improved strength. Although it is not incorporated into tissue and has a high incidence of seroma formation, ePTFE remains inert and produces little inflammatory effects and lower scar density (when compared to PP and PET), which allows it to be placed directly on viscera [68]. Even though minimal inflammatory reaction and lower scar density of this mesh for Intraperitoneal use, this material can be broken easily. Hence the right fixation is quite important [77].

Some meshes mainly used for intraabdominal and occasionally for inguinal hernia repair are made of ePTFE (two-sided DualMesh, IntrameshT1, Dulex, and Composix).

TABLE 13.4 Different commercial meshes based on poly(ethylene terephthalate) (PET).

Type of the mesh based on PET	Characteristics	Applications
Parietex ADP2	It has a lateral slit and prefixed suture so as to encircle the cord structures	Inguinal hernia
EaseGrip	Three-dimensional elliptical shaped mesh; it has a lateral slit with an adjustable self-gripping flap	Inguinal hernia
Parietex ProGrip	Made up of polyester monofilament and polylactic acid is a lightweight self-gripping mesh	Initially devised for use in open Lichtenstein repair, its use has gained popularity in laparoscopic inguinal hernia repair
Parietex ProGrip Laparoscopic	It is made up of monofilament polyethylene terephthalate, which is covered with two layers (the first layer is microgrids made from polylactic acid, which is slow absorbing; the second layer is fast absorbing (in a day), made from a combination of collagen and glycerol)	

From Covidien.

DualMesh coated with silver-chlorhexidine film which acts as an antimicrobial agent is also available [74].

During the last time, meshes prepared from combined materials are sometimes preferred. The main purpose of this mesh type is to prevent the complications by taking advantages of the best traits from two different materials. Thus a microporous mesh permits placement adjacent to viscera, whereas macroporous mesh promotes parietal tissue ingrowth. These meshes can be modified and are easily cut to fit specific applications. These characteristics have also been demonstrated in animal models to decrease visceral adhesions and complications [50]. These properties permit intraperitoneal placement (e.g., DualMesh, Dulex, and Composix). For example, when Composix (a mesh obtained by combining PP with ePTFE) is used, overlap of ePTFE stops adhesions at the edges.

In the case of combined meshes of polyester and PTFE, the former component allows the abdominal wall tissue in-growth, whereas the later one prevents the occurrence of intestinal adhesion, achieved through different pore size of the mesh. A recent movement in the design of combination synthetic meshes is to construct a mesh consisting of a PP or PET base coated with absorbable polymers [68].

PET and PET/chitosan electrospun meshes revealed good performance during incisional hernia surgery, postoperative period, and no evidence of intestinal adhesion was found [78]. The electrospun meshes were flexible with high suture retention, showing tensile strengths of 3 MPa and breaking strains of 8%–33%. Nevertheless, a significant FBR was observed in animals treated with these nanofibrous materials. Animals implanted with PET and PET/chitosan electrospun meshes (fiber diameter of 0.71 ± 0.28 and 3.01 ± 0.72 mm, respectively) showed, respectively, foreign body granuloma formation, averaging 4.2- and 7.4-fold greater than the control commercial mesh group (Marlex).

Many FBGCs involving nanofiber pieces were also found in the PET and PET/chitosan groups (11.9 and 19.3 times more FBGC than control, respectively). In contrast, no important FBR was observed for PET microfibers (fiber diameter: 18.9 ± 0.21 mm).

13.2.2.1 Biologic response to mesh

Incorporation of mesh into tissues is a complicated biochemical healing process. As in the case of other implants, induced biological reactivity will vary according to the weight, effective porosity, size and type of filament, mesh design, presence of coating, and individual host response [79]. Once a mesh is implanted, there is protein adsorption around the prosthesis forming a coagulum by combining together albumin, immunoglobulins, plasminogen, fibrinogen, and complement factors around the mesh material [80,81]. These proteins are known to interact with the cellular components namely platelets, monocytes, macrophages, and polymorphonuclear leukocytes involved in the inflammatory response [82]. A mixture of cells of various origins (> 80%) in the mesh infiltrate positively express CD68, CD8, CD45R0 and vimentin. Cell migration is followed by collagen deposition, with an increase in the type I to type III collagen ratio over time. The majority of tissue ingrowth and strength may be completed 2 weeks after mesh implantation [66]. The concentration of adsorbed proteins depends on the type of prosthetic material and their interaction is complex. Upon adsorption, the surface activates the classic and alternative complement pathways, especially generating factor C5a (a chemotactic factor for inflammatory cells). Then growth factors such as PDGF, FGF, TGF β , insulin-like growth factor, and EGF are activated and play a significant role in the repair of hernia [83]. Expression of FGF and TGF β increases in the presence of mesh materials. Around a week after implanting the mesh material, the population of mononuclear phagocytic cells differentiates into macrophages. These cells secrete a wide number of effectors which modulate the biological response [84]. The inflammatory reaction seals the foreign body in an epithelioid granuloma. In the presence of indigestible prosthetic material, the macrophages coalesce into FBGCs [85]. The final stage of the biological response is the synthesis of connective tissue. The collagen is synthesized and excreted by fibroblasts as monomeric form into the extracellular space where it polymerizes into an insoluble helicoidal structure. A collagen network is produced for around 21 days, then there is an alteration in the ratio of collagen type III and I, that is, there will be a reduction in the level of immature collagen (type III) and a rise in the mature collagen (type I). The three-dimensional (3D) collagen network grows around and through the prosthesis, its mechanical strength increases progressively until ~6 months after performing the surgical wound. The newly formed tissue has only 80% of the normal mechanical strength of the skin or fascia. Other properties, such as elasticity or energy absorption capacity will be even lower. The tissue being weaker and more fragile than normal [86] (Table 13.5).

A detailed comparison between different meshes was made by Zhu et al. [66]. Different synthetic materials behave in unique ways *in vivo* [62,87]. After biomaterial implantation, the host tissue reaction begins with an acute inflammatory response, ultimately leading to collagen production and scar formation. Immediately upon implantation, blood–material interactions lead to the development of a blood-based transient matrix that forms surrounding the biomaterial [88]. The immune response is triggered by injury to vascularized connective tissue. The matrix is rich in cytokines, chemoattractants, and growth factors that

TABLE 13.5 Mesh materials and their biological response [86].

Material	Commercially available meshes	Inflammation	Granulocytes	Fibroblasts	Giant cells	Macrophages	Adhesions	Classification	Tissue in-growth	Resistance to infection
Polypropylene	Marlex	Abundant	Moderate	Moderate	Moderate	Moderate	Moderate	Permanent	Extensive	High
	Prolene	Moderate	Slight	Abundant	Moderate	Mild to moderate	Mild to moderate	Permanent	Extensive	High
Polyester	Mersilene	Abundant	Abundant	Abundant	Abundant	Abundant	Moderate	Permanent	Extensive	May promote
ePTFE	Gore-tex	Moderate	Moderate	Moderate	Moderate	Moderate	Rare	Permanent	Minimal	May promote
	IntrameshT1	Minimal	Mild to moderate	Moderate	Moderate	Moderate	Mild to moderate	Permanent	Allows on PP side	Nil
<i>Synthetic and partially absorbable meshes</i>										
Polyglactin 910	Vicryl	Nil	Nil	Minimal	Nil	Nil	Rare	Partially absorbable	Mild-moderate	Mesh dissolves
Polyglecaprone 25	Ultrapro	Moderate	Moderate	Abundant to slight	Slight/moderate to moderate	Slight/moderate to moderate	Mild to moderate	Partially absorbable	Nil	Nil
Polypropylene	C-Qur	Slight to moderate	Moderate	Slight/moderate to moderate	Slight/moderate to slight	Slight/moderate to slight	Less	Absorbable	Nil	Nil
Polyester	Parietex composite	Moderate	Moderate	Abundant	Abundant	Abundant	Mild to moderate	Absorbable	High	Nil

ePTFE, Expanded poly(tetrafluoroethylene).

enhance cellular activity and the subsequent activation and proliferation of other mediators in the early wound healing response. Histamine-mediated phagocyte recruitment and adhesion to implant surfaces is facilitated by proteins adsorbed in the matrix. Enhanced neutrophil activity along with histamine and IL release from mast cells plays an important role in this subsequent phase [89]. The chronic inflammatory response develops when monocytes and lymphocytes are recognized at the implant site. This leads to eventual FBGCs formation through cells fusion. The process of tissue ingrowth into the mesh is a result of the local inflammatory response upon implantation. This is characterized by macrophage activation and fibroblast proliferation, the activity of which is mediated by cytokines and growth factors [90]. Brodbeck et al. [91] found that surface characteristics of biomaterials (hydrophobic/hydrophilic) influence local cytokine response. The enhanced inflammatory response may have more to do with total mesh surface area than mesh weight itself. They showed that cytokine responses from cells adherent to hydrophobic biomaterials, such as PP, were much more pronounced, whereas hydrophilic surfaces caused lower levels of cytokine production and leukocyte adhesion independent of one another. Di Vita et al. [92] showed that the use of PP mesh for inguinal hernia repair is associated with a higher production of inflammatory cytokines (serum IL-6 and IFN) compared with conventional hernia repair. They found that women with mesh erosion had significantly higher preoperative IFN levels than women with normal vaginal wound healing.

Implantation of the same material in two different anatomic locations—abdomen and vagina—resulted in different histologic responses. Tissues surrounding vaginal grafts had significantly higher scores for inflammation and neovascularization and lower scores for fibroplastic proliferation than tissues surrounding abdominal grafts.

The amount and type of collagen production stimulated by different materials may vary owing to their respective properties and environment [93,94].

Mesh complications, such as erosion, may be linked to bacterial contamination at the time of mesh insertion. A biofilm is an aggregate of microorganisms existing within a self-produced matrix surrounding the implanted foreign body. Biofilms that form after biomaterial implantation can shield bacteria from antibiotics and may lead to subacute and chronic infections. Biofilms can make it difficult for host immune responses to counter the microbial invasion and proliferation. Biofilms provide antimicrobial resistance through delayed penetration of the antimicrobial agent and altered growth rate of biofilm microorganisms.

Mesh characteristics, such as material, pore size, and weave, may not only dictate their biocompatibility, but also the in situ tissue response. Pore size and mesh weight may also influence collagen production and maturation. PP mesh characteristics influencing cellular response are: tensile properties, architecture, pore size, weave of fibrils, weight, surface characteristics, total implantation size, surface area, monofilamentous versus multifilamentous, shrinkage, stiffness, fibril diameter, elasticity, degradation. In general, macroporous PP meshes are preferred due to a more favorable host response. Nonmesh factors influencing cellular response are: anatomical location of implant, exposure to bacteria, individual patient characteristics, persistence of dormant bacteria, presence of bacterial biofilms, combinations with other materials, coatings, release of cytotoxic chemicals. The impact of these degradation compounds on the human is of significant clinical importance. Degradation products may adversely affect exposed humans.

PP meshes result in an intensified inflammatory reaction with deposition of more collagen fibers and significantly higher collagen type I/III ratios within the resulting scar neotissue compared with ePTFE meshes. The increased inflammatory response increases the chances of tissue breakdown, as seen in meshes used to support the uterus and have eroded through the vaginal wall [95]. PET meshes induce the greatest foreign body reaction and longest-lasting chronic inflammatory response, which may be enhanced by the construction of PET fibers as a multifilament. Comparison between PET films and PEO (PEO) modified PET surfaces, implanted in the peritoneal cavity of mice, evidenced that the control PET surfaces showed an initial inflammatory reaction, followed by an extensive fibrotic response, with a mean thickness of 60 μm after 28 days of implantation. By contrast, PEO-modified PET showed only a mild inflammatory response and no fibrotic encapsulation throughout the implantation period, a cellular monolayer being also observed [96]. Apparently, either the PEO-modified surface was stimulating less inflammation, which was in turn stimulating less fibroblastic overgrowth, or the cellular adhesion to the PEO-modified surface was too weak to support cellular multilayers.

Marked fibrosis and encapsulation surround ePTFE films. PTFE is a more reactogenic material than PP, and it primarily stimulates the local production of proinflammatory cytokines. Therefore the local antiinflammatory effect of PP is less pronounced in comparison, but the inflammation persists for a longer time. PVDF meshes produce a significantly reduced foreign body granuloma size compared with PP. PP is less stable than PVDF *in vivo*. Coatings may influence the degree of the inflammatory response. Nonabsorbable and absorbable materials are used for coatings. Absorbable materials are preferred if the coating provides a drug-eluting function. However, the degradation products may also influence the inflammatory response. A comparison of PP meshes, PP + PG meshes, and PP + TI meshes demonstrated a reduced inflammatory reaction in the PP mesh group and increased reaction in the PP + PG mesh group. The PP mesh induced large early elevations in vascular endothelial growth factor, cyclooxygenase-2 and collagen levels, whereas the PP + PG mesh caused only small elevations in the levels of these factors. PP + TI meshes induced inflammatory response levels in between those of the other two meshes. Human fibroblasts colonized on the macroporous PP side of a composite mesh made of two PP layers, but no cell growth occurred on the film PP side. The suppressive effect of the mesh on the TGF β 1 was more pronounced for partially absorbable materials compared with pure PP meshes, which suggests that a change in raw material composition and type affects the early biological reaction of connective tissue cells to the mesh. The textile design markedly influences the inflammatory reaction to the mesh.

The pore size must be much larger than 75 μm to preserve tissue integration without filling the pores with scar tissue. A pore size $>1\text{ mm}$ is required for PP, and the pore size should be $>3\text{ mm}$ in cases of mechanical strain. Meshes with large pores exhibit less inflammatory infiltrate, connective tissue, fistula formation, calcification, and bridging (i.e., the pores are filled by scar tissue) than meshes with small pores. Large pore sizes preserve the “effective porosity” and thus avoid formation of scar bridges. Bridging of granuloma and encapsulation of the entire mesh is more likely for PP meshes with small pores ($<800\text{ }\mu\text{m}$). In contrast, PVDF meshes do not exhibit bridging even for pore sizes of $<650\text{ }\mu\text{m}$. *In vitro* animal studies have shown a significantly greater and prolonged inflammatory response with heavy weight (HW) compared with lightweight meshes.

The coagulum causes platelet adherence, which attracts polymorphonucleocytes, fibroblasts, macrophages, and other platelets by releasing chemoattractants depending on a number of patient factors. An intense foreign body reaction occurs leading to collagen deposition in the ECM. The collagen undergoes transformation from immature to mature collagen increasing its strength gradually till 6 months, achieving 70%–80% of strength of native connective tissue [85,97]. It never completely regains the original strength of native tissue [74]. PP induces an intense biological response causing protein adherence and leading to scar tissue formation. The scar formation also leads to prosthetic contraction. The same response leads to adhesions at the interface between the mesh and host tissue. Intense adhesions and scar formation are common reasons for operative site discomfort and chronic pain after inguinal hernia repair [98,99]. When in contact with intraabdominal contents, for example, bowel these adhesions can lead to intestinal obstruction or fistulization in the worst-case scenario. Hence the need to use composite or meshes with a barrier when used intraabdominally. Properties of the mesh material and individual host response play a vital role [100,101]. The number of granulocytes, macrophages, and fibroblasts served to reflect the tissue response. In regard to the inflammatory infiltrate the remodeling process at the interface the cellular activation was evaluated by TUNEL (DNA-strand breaks or apoptosis, respectively), Ki 67 (cell proliferation), and HSP 70 (cell stress). The measured tensile strength of the low weight (LW) mesh confirmed a sufficient strength of the material-reduced mesh modification. LW PP mesh made purely of monofilaments was compared to a common HW PP mesh in regard to the functional consequences and the tissue response. After implantation the consecutive impairment of the abdominal-wall mobility was reduced compared to the HW mesh, concomitantly to the reduced fibrotic level at the interface. At the end of the observation period the FBR was significantly lowered for the LW mesh with improved biocompatibility, almost reaching physiological values [56,57].

The coating is thought to reduce protein coagulum adherence leading to a reduced inflammatory response. This in turn should reduce mesh to tissue adhesions and hence lower the incidence of chronic pain (see other chapters, e.g., composites). Meshes with anatomical design to conform to the shape of inguinal anatomy were manufactured. 3D Max mesh (Bard Davol, United States) is made up of PP lightweight monofilament with large pore size [74].

13.2.3 Cellular response to synthetic polymers used in cardiac surgery

13.2.3.1 *Poly(vinyl chloride), poly(tetrafluoroethylene), poly(urethane), and poly(ethylene) as catheters in cardiac surgery*

The most commonly used catheter is made of medical-grade plastic, such as PVC or silicone. Single-use catheter (PVC) appeared safe and effective [102]. Hydrophilic catheters are PVC catheters coated along the entire length with a hydrophilic polymer, primarily poly(vinyl pyrrolidone) (PVP) and same with sodium chloride. When these catheters are exposed to water, the PVP coating attracts the water to the surface of the catheters, creating the biocompatible salt coating that binds the water to the surface of the catheter and forming an outer layer mainly consisting of water. This thick, slippery, smooth layer of

water stays on the catheter, ensuring lubrication of the entire urethra during catheter insertion and withdrawal, thereby reducing the friction coefficient by at least 95% [103]. PVP is a nonallergenic substance. Because of their low friction, PVP-coated catheters seem to be associated with a lesser degree of urethral inflammatory response when compared to PVC catheters [104]. However, there is an increasing demand for PVC-free materials, with efforts to avoid the use of medical devices that use PVC and its plasticizer [di (2-ethylhexyl) phthalate] [105].

Usually, central venous catheters develop two complications, catheter-related infections and occlusion, particularly by thrombus. Catheter-related bloodstream infections (CRBSI) cause nosocomial infections that increase patient morbidity, mortality, and hospital cost. Occlusion of a catheter due to fibrin sheath formation or thrombosis renders the catheter unusable, its replacement being necessary. Thrombus forms consist in platelet aggregates and crosslinked fibrin because of cellular and protein-cell interactions. The artificial surfaces have no directed activity against adherent proteins or cells, no remodeling capabilities, and no active mechanism to enhance or inhibit cell signaling. Therefore a multistep process of protein adsorption, cellular adhesion, thrombin generation, and activation of complement occurs at the surface which creates thrombotic occlusion. The major development steps of thrombosis on a catheter surfaces are: (1) Protein (fibrinogen, Fn, vWF) adsorption, with increased protein concentration (Vroman effect); (2) Cellular adhesion as: platelets; red blood cells (RBCs); neutrophil extracellular traps (NETs) with activation and release of agonists such as thromboxane A₂ and ADP; (3) Platelet aggregation (signaling molecules, platelet-near wall effect, enlargement of aggregate); (4) fibrin crosslinking (stabilization of platelet aggregate, intrinsic pathway). Cellular adhesion activates cascading effects to promote aggregation [106]. Adsorption is reversible and can change the local concentrations of adsorbed proteins (Vroman effect). Hydrophilicity is a key determinant of protein adsorption. Hydrophobic surfaces adsorb proteins more readily. A key component in thrombosis is fibrinogen and it is likely one of the first components to adsorb to artificial surfaces. Fn, vWF, and fibrinogen mediate platelet adhesion. The complement system and proteins coagulation activated in highly concentrated environment facilitating thrombosis. Fibrinogen is the major factor in platelet aggregation on artificial surfaces [107]. As platelets aggregate to the adsorbed fibrinogen, they release thromboxane A₂, ADP, and other agonists which activate further aggregation. Fibrinogen will also attach to leukocytes and create an inflammatory state that increases platelet aggregation via multiple signaling molecules such as platelet activating factor and TNF. RBC adhesion is passive but once adhered can release ADP which stimulates platelets [107]. An increased concentration of platelets at the periphery increases the platelets interactions with the catheter surface developing thrombus. PTFE and PUR catheters are associated with decreased rates of CRBSI when compared to PVC and PE [108].

Biofilm-related infections are extremely difficult to eradicate due to high tolerance of the biofilm to antibiotics and resistance to the host immune systems [108]. Stabilization occurs via fibrin crosslinking. Artificial surfaces can exacerbate portions of these steps [109,110]. Thrombus contains complicated arrays of histone/DNA complexes when it is in the acute phase. Local inflammation developed around thrombus creates NETs, an array of DNA, and proteins with the intent of antimicrobial activity. These can then provide a scaffold with altered flow for platelet adhesion and further propagation of the thrombus.

The catheter impregnation with antibiotics, nanoscale surface design, are innovative changes to improve the intravenous catheters. Heparin (HEP) also has been used as a coating agent to prevent infectious fouling. The immobilization of sodium alginate/HEP reduced the contact angles of the coated surface, protein adsorption and adhesion, improve the antithrombogenicity, platelet adhesion and thrombus formation were reduced, activated partial thrombin and thrombin time of the coated PVC was significantly prolonged as compared with the noncoated PVC. By the immobilization of HEP, biocompatibility and hemocompatibility, which are essential for cardiac pulmonary bypass surgery are improved [111]. Adsorbed proteins can form a layer 2–10 nm in thickness and enhance the concentration of these proteins 1000 times higher than in plasma.

Local leakage of toxic substances is continuous and can results in persisting inflammation, cell damage, and death [112].

13.2.3.2 Poly(ethylene terephthalate) and poly(tetrafluoroethylene) used in cardiac surgery

The materials used in cardiovascular applications for prosthetic heart valves, catheters, heart assist devices, hemodialyzers, synthetic vascular implants and stents have to meet the requirements for biocompatibility/hemocompatibility and should also have appropriate mechanical properties, in particular the flexibility and ease of surgical implantation [113]. Today, the following polymers are used for this purpose; PA, polyolefin, polyesters, PURs, PET, and PTFE [114]. Even if all these materials were used for synthetic vascular prosthesis for many years, unfortunately they do not offer sufficient hemocompatibility, especially when used for replacement of veins of smaller diameters (<6 mm). The main reason for this is that the probability of thrombosis occurrence is even greater in the narrower part of the veins. On the wall of the artificial vein, there is a nonspecific binding of plasma proteins, which also affects the platelet binding and is one of the main causes of thrombosis [115]. Lack of endothelializations is another main cause of thrombosis.

At the beginning, the most suitable materials for this application type were inert materials that do not react with the body and do not allow their integration with the body. Today, the opinion is that biocompatible materials in contact with blood should enable interaction with the body and prevent infections, inflammatory reactions, blood clotting, and other related reactions [116].

Over the years, different methods for adequate modification of the polymer surfaces used in cardiac surgery were used (i.e., chemical treatments, irradiation with photons, irradiation with ion or electron beams, plasma treatment, etc.), together with the development of materials that mimic the properties of natural cardiac tissues, that is, composite materials [116–119]. In biomedical applications, the best results are most commonly achieved by the use of oxygen-containing plasma, which was found to improve hemocompatibility of polymer grafts, by making the polymer surface antithrombogenic, because of reduced platelet adhesion [120]. Furthermore, oxygen plasma also improves cell adhesion and proliferation [121–130,215].

Laser treatment applied to PET surfaces designed for being used in vascular prostheses, was found to improve cell proliferation (up to 140% with respect to controls), vitality (10% higher than controls), morphology, and adhesion kinetics (more than 16% of control) [131].

A surface wet-chemistry protocol was applied to graft track-etched PET membranes with arginylglycylaspartic acid (RGD) peptidomimetics based on the tyrosine template and active at the nanolevel versus. isolated human $\alpha_v\beta_3$ receptor [132,133]. It was found that grafting of peptidomimetics as ligands of the $\alpha_v\beta_3$ integrin could be a relevant strategy to improve the adhesion of human ECs (HEC) and to obtain an efficient endothelialized PET for the surgery of small-diameter vascular prostheses.

Due to PTFE inertness (insoluble in all common solvents), high thermal stability, and nonbiodegradability, it has been used in various commercial, industrial, and biomedical applications, including large blood vessel repair material [134]. Apart from this, PTFE has been also used as a graft material, such as in superficial femoral occlusion and left ventricular assist device. PTFE has been found to elicit mild to moderate inflammatory response *in vivo*. After implantation of ePTFE in unilateral aortofemoral bypass of dog, chronic inflammatory response was observed along with the presence of macrophages, myofibroblasts, and deposition of complement C3 after 6 months of implantation [8].

Fibroblasts respond to vascular PTFE prostheses by forming a thick external fibrotic capsule and a thin layer coating the pores [135]. Despite an initial quick, spontaneous endothelialization process, complete endothelialization of the lumen of a PTFE prosthesis can take up to 6 months because of the rough surface, which, compared to PURs, makes EC proliferation more difficult. Chronic proliferation is, however, significant. After 6 months, the cells are still dividing, increasing neointimal formation and thus the risk of hyperplasia and thrombosis. Surface texture also influences cell migration. Cell migration, which can prevent thrombosis and occlusion, is slow on PTFE [136].

Bypass surgery for atherosclerosis is confronted with the absence of ECs in the lumen of vascular prosthesis in humans. Implantation of unilateral aortofemoral bypass with ePTFE in dogs demonstrated significant differences between humoral and cellular responses [137]. The area of proximal anastomosis revealed the presence of fibroblasts, but no macrophages were detected. The histological structure of the proximal anastomosis indicates that inflammatory processes were ended during the prosthesis healing. The immunological response obtained in the distal anastomosis corresponded to the chronic inflammatory reaction with the presence of macrophages, myofibroblasts, and deposits of complement C3.

Healing in humans consists in outer wall fibrous tissue and luminal coverage with more or less compacted fibrin only. Incomplete healing of synthetic nonresorbable vascular prostheses imposes a risk of thrombotic occlusion, particularly in medium-diameter or small-diameter blood vessels. One of the most effective methods used for obtaining non-thrombogenic surfaces is artificial EC seeding prior to implantation [138].

Chlupac et al. tried to obtain the endothelialization of knitted PET prostheses [139], which are produced by companies like: *VUP Joint-Stock Co.*, Brno, Czech Republic; *Vascutek*, a Terumo Company; *Lemaitre Vascular, Inc.*; *Bio Nova International Pty Ltd*, North Melbourne, Australia; *Atrium*, Maquet Getinge Group, Hudson, New Hampshire, United States. However, the inner surface of knitted PET vascular graft is rough and irregular, with relatively large void spaces among the PET fibers, for this reason being not suitable for the adhesion, spreading, and growth of ECs, which prefer smoother surfaces. In addition, in its pristine unmodified state, PET is a highly hydrophobic polymer, having the sessile water drop contact angle higher than 100 degrees, which is not suitable for adhesion and growth of cells [140]. The method used by this research group for modifying the knitted PET fabrics

in order to obtain a successful endothelialization was the impregnation with other polymers (synthetic or absorbable) and coating with bioactive molecules.

Between the most promising candidates for impregnation of PET prostheses are absorbable aliphatic polyesters, namely poly(L-lactic acid) (PLLA), poly(D,L-lactic acid), PGA, poly(ϵ -caprolactone) (PCL), and their blends. Absorbable polymers act as temporary templates for the regeneration of vascular tissue. They induce minimal chronic inflammatory foreign body reaction and they also meet two important but contradictory requirements: low bleeding permeability at the time of implantation and high cell–tissue permeability for better full wall healing [139]. In bicomponent vascular graft design, the nonabsorbable fibers that provide strength and the absorbable components are advantageous for tissue regeneration, mechanical properties, and for reducing the foreign body reaction [141].

The chronic inflammatory response associated with the abluminal surface of polymeric vascular grafts has been suggested to affect adversely graft neovascularization, the cellular response at the luminal surface of vascular grafts, and overall graft patency. To better understand the source for this chronic inflammation, Hagerty et al. examined two types of macrophages and the amount of cellular proliferation around two widely used graft materials, ePTFE and PET, Dacron in commercial form implanted in rats, for 3 and 5 weeks [142]. The obtained results showed that Dacron is more inflammatory than ePTFE and that there is a segregated macrophage response; the first 54 μm of perigraft tissue was composed predominantly of recruited macrophages (ED1 +), while the more distal tissue consisted of resident macrophages (ED2 +). Proliferating cells were located predominantly in this same 54 μm perigraft region. In subcutaneous tissue they accounted for 23% of all cells present around Dacron after 3 weeks of implantation and 8% after 5 weeks. Conversely, cellular proliferation around ePTFE increased from 4% at 3 weeks to 21% at 5 weeks. In adipose tissue, proliferation levels around the implanted polymers were lower and more similar after 3 and 5 weeks.

13.2.4 Cellular response to poly(methyl methacrylate)

Panahi-Bazaz et al. compared primary implantation of foldable hydrophilic acrylic poly(hydroxyethyl methacrylate) backbone and hydrophilic acrylic monomers with PMMA intraocular lenses (IOLs) in pediatric cataract surgery [143]. Hydrophilic acrylic IOLs are soft and have excellent biocompatibility because of their hydrophilic surface and 18%–38% water content. IOLs of these polymers show little or no surface alterations or damage from folding because of their soft flexible surface. Low surface energy and hydrophilic nature are major reasons for good uveal biocompatibility. Hydrogel IOLs seem to have lower capsular biocompatibility as compared to other biomaterials. Postoperative complications including pigment deposition, iridocorneal adhesions, and posterior synechiae formation, were seen only in the PMMA group.

13.2.5 Cellular response to poly(urethane) and poly(amides) (nylon)

PURs share the common polymer backbone structure, which includes aliphatic or aromatic units coming from the isocyanate monomers and a more complex moiety derived

from polyether or polyester monomers. PUR has been extensively investigated as a material of choice for long-term cardiovascular medical devices, such as cardiac pacemakers and vascular grafts due to their moderate blood compatibility and mechanical properties [144]. However, they have been shown to elicit an increase in the release of chemokines, cytokines, and growth factors in the *in vivo* models [145]. Subcutaneous implantation of lysine diisocyanate-based PURs in rats revealed that it did not aggravate capsule formation, accumulation of macrophages, or tissue necrosis [146].

Because surface coating of a biomaterial may reduce leukocyte adhesion (biofouling) and can adversely affect immune response [8], preadsorption of less inflammatory proteins (albumin) on PS and PUR surface could be used previously of their medical applications [147,148].

PUR (Tecoflex), surface modified with the nonionic surfactant Tween80 (PUR/T80) and the cell adhesive poly-L-lysine (PLL)–RGD peptide (PUR)/PLL–RGD) were implanted in the peritoneal cavity of Wistar rats for 30 days [149]. PUR/PLL–RGD followed by the bare PUR surface exhibited severe inflammatory and fibrotic response with an average mean thickness of 19 and 12 μm , respectively, in 30 days. In contrast, PUR/T80 surface showed only a cellular monolayer of 2–3 μm in thickness, with a mild inflammatory response and no fibrotic encapsulation. The PUR/PLL–RGD peptide-modified substrate promoted an enhanced rate of macrophage cell fusion to form FBGC, whereas FBGCs were rarely observed on Tween80-modified substrate. The expression levels of proinflammatory cytokines (TNF- α and IL-1 β) were upregulated on PUR/PLL–RGD surface followed by bare PUR, whereas the cytokine expressions were significantly suppressed on PUR/T80 surface.

PURs are characterized by excellent physical properties, adequate for being used in the artificial heart, pacemaker leads, and catheters. One of these, Biomer, a segmented poly(etherurethane urea) (PEUUR), is known to have excellent Hemocompatibility [150]. Vascular grafts made from PURs and having a diameter <6 mm have however generally failed to remain patent following long-term implantation [151].

The advantages of using a material with a mesh structure were first reported by Voorhees et al. [152]. Annis et al. [153] designed a small-diameter arterial prosthesis using the PEUUR Biomer, that it was electrostatically spun to produce cylindrical tubes of randomly-orientated PEUUR fibrils of about 1.0 μm diameter. Porous surfaces have been found to encourage tissue ingrowth, the size of the pores being an important determinant of ingrowth of tissue and maintenance of the inner lining of a vascular prosthesis [154,155]. Starting from this finding, Mohqty et al. prepared vascular prosthesis with a porous internal structure varying from small pores to much larger irregular ones [156]. The pores were interconnected and open to both the internal and external surface of the prosthesis. The extent of the cellular response in general, and especially of the macrophage response, was clearly dependent on the pore microstructure. A pore size in the region 5–10 μm resulted in little infiltration and generally low number of cells in the surrounding tissue, while large pore size in some regions of the wall of the prosthesis (about 200–300 μm) led to a considerably greater cell number in the tissues surrounding the sample.

Considerable effort has been devoted to the development of synthetic substrates that can provide biomimetic signals to cells, encouraging them to attach and behave as they would in their natural environment [157–159]. Efforts of this type could ultimately enable

valuable medical technologies, such as the *ex vivo* growth of tissues that can be used for transplantation. Polymers in the nylon-3 family contain subunits derived from β -amino acids, which are linked to one another via amide bonds. Thus the nylon-3 backbone is homologous to the α -amino acid-based backbone of proteins. This molecular-level homology suggests that nylon-3 materials might be intrinsically protein-mimetic, with clear perspectives for being used in applications concerning tissue engineering [160].

Since the sutures in the oral cavity are exposed to constant bathing with saliva, oral microorganisms, and masticatory trauma, the possibility always exists that a secondary infection may be established at the suture sites. Such an infection might progress along the suture tracts and produce a local inflammatory response in the lamina propria of the gingiva. The tissue reactions of the gingiva to one of the most commonly used suture materials in dentistry (i.e., nylon) were assessed [161], after 14 days of implantation in the tunica propria of the gingiva. After 4 days postoperatively, the reacting cellularity around the nylon tract consisted mainly of young connective tissue cells and occasional histiocytes. Newly formed collagen was also present. Fourteen days of implantation led to a more compact cellularity around the nylon, and the stratification of cells and new collagen indicated the formation of a pseudocapsule. All the obtained results evidenced that nylon sutures did not provoke an inflammatory response.

13.2.6 Cellular response to poly(styrene)

13.2.6.1 *Poly(styrene) as a cell culture material*

PS has served as the fundamental substrate for adherent animal and human cell culture for more than 50 years, the two-dimensional (2D) tissue culture PS (TCPS) being the basic platform for adherent cell culture [162]. Main forms of PS used in biomanufacturing and cell-based research activities are: injection molded, embossed, cast, electrospun and, more recently, 3D printed polymer [163].

Because phenyl groups in PS do not readily provide anchoring points for cells as they are not normally expressed in the human body, this polymer had to be modified in order to facilitate cell anchorage in vitro by incorporating surface functionality (carbonyl and amine groups), which cells will bind to and grow on. Transforming native PS surfaces to include chemistry other than phenyl groups can increase the hydrophilicity and surface charge, modulating the deposition of extracellular matrix, cells, and proteins [14,164,165].

Functionalization methods used for manufacturing TCPS [166] can be broadly divided into two groups: liquid phase and plasma-based treatments.

13.2.6.2 *Surface functionalization by liquid treatment*

The first proposed mechanism for modifying the surface of PS in order to facilitate cell adhesion was introduced in 1966 by sulfonating the surface, with subsequent neutralization with sodium carbonate and water [162]. Sulfuric acid treatment (20% v/v) not only aids in cell binding, but can potentially facilitate adhesion of proteins, such as Fn and vitronectin [167]. The adsorption of these proteins can mediate cell adhesion, spreading, and growth [165,168]. Other acids (e.g., nitric and hydrochloric) can be also used for this purpose, but they also tend to degrade surfaces, reducing the optical clarity [167].

Simulated body fluids can be used to facilitate growth of hydroxyapatite crystals on PS surfaces, inducing thus osteogenic differentiation of MSCs [169], but these ones can be easily removed from the surface.

Full liquid immersion provides a direct method to introduce a number of different surface functionalities (e.g., $-\text{CH}_3$, $-\text{NH}_2$, $-\text{SH}$, $-\text{OH}$, and $-\text{COOH}$), by selecting modifying liquids [170]. This method has the disadvantage of eroding complex surfaces and printed geometry during modification, but complete internal surface coverage is ensured.

13.2.6.3 Surface functionalization by plasma treatment

The majority of research has involved modifying surfaces to incorporate oxygen- and nitrogen-containing species, with the objective of creating surface chemistry that encourages cell adhesion, proliferation, and functionality [171,172].

The binding of extracellular matrix to a plasma-deposited amine surface regulated the interaction and subsequent attachment of human MSCs [173]. Depending on the treatment conditions (discharge gas, exposure time, plasma source power, etc.), surface chemistry can be controlled in terms of increasing cell attachment, growth, and viability [174–176].

In cases where site-specific surface modification is required, plasma jets can be directed through a shadow mask [177,178] or surfaces can be partially covered with photoresist resin [179] to provide spatially distinct regions for surface modification.

13.2.6.4 Surface functionalization by other methods

The previous mentioned methods for modifying PS surface can be followed by advanced grafting techniques (such as self-assembled monolayers [180] or polymer brushes [181]). Grafting of poly(*N*-isopropyl acrylamide) to TCPS surfaces has successfully released adherent culture cells, without introducing additional enzymes, by inducing a conformational change in the polymer brushes as the culture temperature passes below the lower critical solution temperature [182]. Plasmas containing argon and/or oxygen have been used to aid the grafting of additional chemical species, such as *N*-vinyl-2-pyrrolidone, in order to improve biocompatibility, adhesion, and proliferation of L929 cells [183]. DNA has been grafted to PS surfaces using secondary amines [184], a technique that could be translated to antibodies as well [185,186]. Additionally, glucose has been sequestered to PS surfaces using thiolene “click” chemistry [187], a mechanism which could be further investigated for advanced surface functionalization.

The design of polymer surfaces can be used for controlling cell behavior [188]. The differences in the morphological behavior between fibroblasts cultured on nanogrooved (groove depth: 5–350 nm, width: 20–1000 nm) and smooth PS substrates were analyzed in detail in order to clarify to what extent cell guidance occurs on increasingly smaller topographies [189]. It was observed that fibroblasts do not align on surfaces with groove depths below 35 nm or ridge widths smaller than 100 nm. The effect of other patterns, such as pillars and pits, has also been analyzed [190], with cell proliferation thought to be increased with decreasing pit diameter [191] and cell attachment thought to be larger on microscale pillars than on macroscale pits [192].

Nanotextured PLLA/PS films stimulated greater osteoblastic cell adhesion and spreading than did flat control films; the adhesion and spreading was enhanced on surfaces with a nanoisland topography [193].

Ultrathin films of diblock copolymers based on PS/poly(4-vinylpyrindine), assembled on mica to form heterogeneous surface patterns [194], evidenced that the size and the morphology of these nanopatterns (which contain dot-like, worm-like, or both features) depend essentially on the relative length of the blocks. Nanopatterns of heights less than 10 nm elicit distinct rates of adhesion and proliferation in fibroblasts and mesenchymal precursor cells, although both cell types adhered and proliferated better in the worm-like, coated substrate [195]. The distinct cell types also affected the extracellular matrix environment differently during their migration [195].

Jeon et al. evidenced that, among various physicochemical properties (such as: aggregation/agglomeration, protein corona formation, and compositional elements), the surface charge of nanoparticles is one of the key parameters that decides their biological impact [196]. Thus in the lung inflammation model, the value of zeta potential showed a good positive correlation with the inflammogenic potential of metal-oxide nanoparticles or PS nanoparticles [197,198]. In their study, they synthesized fluorophore-conjugated PS nanoparticles (F-PLNPs), with different types of surface functional groups, all based on an identical core. Phagocytic differentiated THP-1 cells or nonphagocytic A549 cells were incubated with F-PLNP for 4 hours, and their cellular uptake was quantified. The amount of internalized F-PLNPs showed a good positive correlation with the zeta potential of F-PLNPs in both cell lines (Pearson's $r = 0.7021$ and 0.7852 for zeta potential vs cellular uptake in THP-1 cells and nonphagocytic A549 cells, respectively).

13.2.7 Cellular response to other synthetic polymers

Poly(sulfone) (PSf), PEEK, and PEI are thermosetting polymers widely investigated for biomedical applications. These engineering polymers are characterized by high mechanical properties, thermal stability, very marginal water absorption, and relatively easy processing. In addition, their high level of solvents and thermal resistance allows the production of sterilizable medical devices [199].

13.2.7.1 *Poly(sulfone)*

Poly(sulfone) (PSf) is a polymer that has properties matching those of light metals, showing outstanding oxidation and thermal degradation resistance, presenting good impact strength and ductility with high elongation to break and tensile strength. Moreover, polysulfone exhibit excellent resistance to hydrolysis or reduction of molecular weight even at elevated temperatures. However, the wear properties of this material are not as good as PE and polyoxymethylene [200]. The PSf-based membrane has been increasingly used in many industrial and medical fields, due to its good chemical and temperature resistance, as well as good mechanical strength and stability. The PSf membranes also showed high permeability for low-molecular weight proteins when used for hemodialysis. In the “non-self” HD application, PSf-based membranes exhibit the intrinsic biocompatibility, high permeability for low-molecular weight proteins, high endotoxin retention, and high resistance during sterilization [201,202]. In addition, the PSf membrane meets the solute and fluid removal requirement for all treatment modalities (low- and high-flux dialysis, online hemodiafiltration, and hemofiltration) [203]. Despite these advantages, the PSf membrane

faces some limitations when it is in contact with the human blood during the HD process. The HD membranes are limited with progressive flux decline and changes of membrane selectivity due to *fouling*. This fouling is mainly attributed to the accumulation of protein, which could be followed by the activation of different defense systems in blood [157,204]. Therefore further modification of the PSf membrane is required to maintain their sustainability on the subject of biocompatibility [205]. Thus, all the PES membranes used for hemodialysis are not the pristine PES membranes, and most widely used modification method for hemodialysis PES membranes is blending. PVP is the most widely used for the modification of PSf membranes by blending, and PVP also acts as a hydrophilic additive and a membrane forming agent. Surface-coating and grafting methods can also be used for the modification of PSf hollow fiber membranes. All the modifications are based on the premise that the materials used in the modification give inherently more hydrophilicity and adsorb less protein than the underlying substrate. Protein adsorption on material surface is a common phenomenon during thrombogenic formation. Thus the amount of protein adsorbed on the PES membrane is considered to be one of the important factors in evaluating the blood compatibility. The adhesion of platelets to blood-contacting medical devices is a key event in thrombus formation on material surface [206].

13.2.7.2 Polyethersulfone

PES is a parent material of PSf, with a better chemical resistance, thermal stability, mechanical properties, as well as a better hydrophilicity compared to PSf [201]. Animal and clinical experiments indicated that the PES-based high-flux hemodialysis membrane had good blood compatibility, and could effectively remove “middle” molecular solute as β_2 -microglobulin. The PES and PSf membranes showed similar blood compatibility and solute clearance [206]. Activation of blood cells during hemodialysis is considered to be a significant determinant of biocompatibility of the hemodialysis membrane because it may affect patient health adversely through microvascular inflammation and oxidative stress [207]. An *in vitro* study performed by Koga et al. [207] assessed the effects of different polysulfone hemodialysis membranes on blood cells, and showed that the number of platelets adherent to their surfaces, and ROS production by neutrophils were clearly different among membranes. For example, a polysulfone membrane with a thickness of swollen layer of 5.3 nm induced adhesion of many platelets and increased the surface expression of activated CD11b and ROS production of neutrophils; however, other polysulfone membrane with a thickness of swollen layer of 10.4 nm had virtually no effect on platelets and neutrophils. The adhesion of platelet and ROS production by neutrophils were mediated by antiintegrin GPIIb/IIIa antibody on platelets, and macrophage-1 antigen (Mac-1) and $\alpha v\beta 3$ on neutrophils, respectively. In addition, the number of adherent platelets and neutrophil ROS production has risen with the amount of fibrinogen adsorbed on the membranes. These results suggested that fibrinogen adsorbed on PSf membranes induced GPIIb/IIIa (platelet membrane glycoproteins) mediated platelet activation and Mac-1 and $\alpha v\beta 3$ -mediated neutrophil activation, depending on the amount of adsorption. The authors conclude that a membrane with lower fibrinogen adsorption may reduce cell activation during dialysis, and subsequent microvascular inflammation and oxidative stress during HD treatment. Su et al. [201] conducted a clinical study regarding the evaluation of blood compatibility of PSf and PES hemodialyzers. Slight neutropenia and platelet adhesion were observed at the initial stage of the hemodialysis and no

significant difference was found in electrolyte or blood biochemistry before and after the treatment. They noticed a slightly decrease in outlet leukocyte counts that was caused by complement activation, thus similar results were obtained in the changes of complement factor C3 and complement C4 during the hemodialysis process. The protein adsorption for pure PSf was higher than that for PES because the hydrophilicity of PES and PSf was different. Slight changes in total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were also observed. There are no significant differences in the changes of TBIL, DBIL, ALT, and AST for the PES and PSf membranes, and both the TBIL and DBIL levels increased compared to the initial levels, which were presumably caused by the dilution of the blood by normal saline solution infused or pachemia after the hemodialysis process. Their results indicated that the PES and PSf membrane had no effect on the liver.

13.2.7.3 Poly(etherimide)

PEI materials exhibit numerous favorable characteristics for biomedical purposes. PEI-based materials possess high mechanical strength, thermal stability, easy processability and chemical resistance allowing different sterilization methods (γ -rays and autoclave), which is a prerequisite for clinical applications [7]. With regard to biocompatibility, PEI materials exert minimal cytotoxicity, good hemocompatibility (no hemolysis), are immunocompatible and have shown promising features as an excellent substrate for cell spreading and growth [199,208–211]. Based on these considerations, PEI membranes have emerged as suitable materials for blood-contacting applications, such as blood detoxification and oxygenation [7,208]. However, solid tissue-contacting applications, for example, drug delivery and wound healing systems, biohybrid organs or neuroprostheses, are also conceivable [212,213].

Peluso et al. [214] have tested PEI biocompatibility both *in vitro* and *in vivo* and noticed that even if PEI induces FBCGs formation *in vivo*, it seems to determine only a moderate degree of hydrogen peroxide production by adherent leukocytes. The cytokines, produced in large amounts during the inflammatory processes, would promote the formation of FBCGs leading to an increased number of FBCGs or an increase in the size of the FBCGs already present on the material surface. The observation that only at 21 days *following cage implantation* was it possible to show a significant increase in FBCG numbers on the PEI surface may be evidence for the presence of *mild inflammatory response* to the material implant. In fact, previous studies have demonstrated that the inflammatory stimulus, due to the presence of reactive material in a tissue, is an important factor controlling FBCG formation. They used the hydrogen peroxide-sensitive dye 2',7'-dichlorofluorescein diacetate to examine the functional activity of cells adherent to PEI or control material implants. PEI material did not induce significant DCFH oxidation until day 14, when levels of DCFH oxidation were significantly higher than observed with polydimethylsiloxane. It was possible to grow on PEI surfaces not only 3T3 fibroblasts but also HEC from human umbilical cord, with no apparent deleterious effects on the cell viability [214].

Surface topography, porosity, surface:volume ratio and local mechanical differences considerably influence the tissue response to a foreign material. Furthermore, different processing methods (melt vs solvent) might also have an impact. Based on the last mentioned considerations the article of Haase et al. [7] presents an *in vivo* biocompatibility study regarding the impact of basic material topography on the implant reaction. For that

purpose they subcutaneously implanted PEI polymers in the form of films prepared via melt extrusion and porous, fibrous scaffolds obtained by solution electrospinning, and evaluated typical parameters of the foreign body reaction 7 and 28 days after implantation. In contrast to PEI films with a smooth surface, the scaffolds showed a decrease in capsule thickness and inflammatory cells infiltrating the capsule during the observation period. The textile behavior and high porosity of the PEI scaffolds enabled them to fold and comply with the host tissue, and allowed for the rapid cell ingrowth, production of extracellular matrix components and vessel formation promoting tissue integration.

13.2.7.4 Poly(etheretherketone)

PEEK is a polyaromatic semicrystalline thermoplastic polymer with thermal and mechanical properties favorable for biomedical applications. It was proposed as a material for biomedical application in 1998 by Invibio Ltd. (Thornton-Cleveleys, United Kingdom) and in the same year Victrex PEEK business (Imperial Chemical Industry, London United Kingdom) launched PEEK-OPTIMA and later carbon fiber reinforced (CFR) PEEK-OPTIMA for long-term implantable applications. These compounds and composites have undergone extensive biocompatibility testing to meet the criteria for their FDA Master Files [215]. At the moment the medical application of PEEK materials is common in several surgical fields, and a possible classification regarding clinical application includes: (1) PEEK for bone replacement-maxillo-facial and cranial implants; (2) PEEK for spine surgery-spinal cages; (3) PEEK for orthopedic surgery for bone and hip-replacement-articulation, implants; orthopedic devices from PEEK material—fixation plates, screws; (4) PEEK for tooth replacement—dental implants from CFR-PEEK, dental prosthesis, intraradicular posts; (5) PEEK for cardiac surgery—intracardiac pump, heart valves [216]. Obtaining of PEEK composites broadens the physicochemical and mechanical properties of PEEK materials. To improve their osteoinductive and antimicrobial capabilities, different types of functionalization of PEEK surfaces and changes in PEEK structure were proposed. Over time different experiments were performed to evaluate the PEEK biocompatibility. Neat PEEK and carbon fiber reinforced PEEK samples were subcutaneously implanted in rabbits for 6 months and submuscularly implanted in rats for 30 weeks and have elicited a “minimal response” in both animal models [217]. The growth and attachment of osteoblasts and fibroblasts to PEEK was evaluated by Hunter et al. [218] in a series of cell culture experiments. Cell lines were obtained from rat osteogenic sarcoma (osteoblasts); rat tail tendon (fibroblasts); and human fetal lung (fibroblasts). The results of their study suggested that PEEK did not appear to deleteriously affect osteoblasts and fibroblasts. Similar conclusions were drawn by Morrison et al. [219] when testing PEEK on fibroblast cell lines derived from the 3T3 mouse and osteoblasts derived from neonatal rats. The authors found that PEEK was not cytotoxic and suggested it can be considered in the development of isoelastic hip stems. Tests (Ames test) performed by Katzer et al. [220] confirmed that PEEK is not mutagenic. Jockisch et al. [221] implanted CFR-PEEK into rabbit muscle over 8–12 weeks and observed normal muscle tissue with *no adverse tissue response* and *no visible infection* with only a few *inflammatory cells* when analyzed histologically. The tissue response was comparable to UHMWPE. The researchers’ second phase study used CFR-PEEK as internal fixation devices for transverse midshaft femoral

osteotomies in canines and demonstrated the material to be effective in promoting fracture healing with a nonspecific FBR shown to plates and particulate debris [215].

The results obtained by Trindade et al. [222] demonstrate the immune system activation around PEEK implant once in contact with host bone, after 10 days of implantation, demonstrating an immune activation. Also, PEEK are still dealing with a mixed proinflammatory M1-macrophage (M1) and M2 antiinflammatory type of reaction (CD68, CD14 and CD11b; ARG1, respectively). The conclusion was based on the fact that the macrophage polarization, between M1-macrophage and M2-macrophage phenotypes, has been highlighted as a determining factor in the foreign body reaction, that is, how host tissues interact with biomaterials. M1 macrophages present a proinflammatory phenotype, while M2 macrophages have been identified as antiinflammatory cells, participating in wound healing, namely in the healing phase of acute inflammation, and also in chronic inflammation associated with immunological diseases, such as rheumatoid arthritis and psoriasis [223]. The experiments also showed activation of CD4 + T-cells around PEEK material at 10 days, whereas the CD8 + T-cell phenotype is suppressed. These findings demonstrate the participation of T-cells in the bone healing process around solid biomaterials, although it is not known whether solely an innate or also an adaptive type of immune reaction is present. Classically, the host reaction to biomaterials is perceived as an innate immunological process, hence indicating T-cell activity through cytokines, rather than an antigen–antibody interaction. Complement factors seemed mostly suppressed around all of the materials studied, at 10 days [222].

13.3 Cellular response to biodegradable/resorbable polymers

Over time efforts have been made to develop biodegradable polymer materials, which can be broken down by intracellular processes and rapidly eliminated from the body. Although biologically derived biodegradable polymers such as polysaccharides and proteins have good bioactivity and accessibility to cell-triggered proteolytic degradation, most of these polymers face practical limitations including a robust immunogenic response and the complexities related to their chemical modifications. Thus synthetic biodegradable polymers have become important alternatives for bioapplications [224].

Performing *osteosynthesis* with the implantation of a metallic device inside the body sometimes promotes development of an infectious process that, in the past, has even led to amputation of the infected limb. Infection can arise a long time after the end of the healing process, so, bearing this in mind, maintaining a metallic device for a longer time, or even for life, is not recommended. However, the rule of removing an *osteosynthesis* device after completion of the healing process is not easy to implement for two main reasons: the first is that in several parts of the body the second operation required to remove the implant may be difficult and risky; the second is that it is costly. The idea of having devices made of *biodegradable polymers* that were able to sustain the mechanical load during the healing process of a fracture but, afterwards, would gradually disappear from the site, was greeted with great enthusiasm in the early 1990s [2]. Even if the using of biodegradable materials in orthopedic surgery has overcome many disadvantages associated with metallic implants, the biggest disappointment came from the discrepancy that exists between the biological and clinical

acceptation of “degradation.” There is a discrepancy between what is “degradable” from a chemical point of view and what can be considered “degradable” from a clinical point of view. The “degradation” or “resorption” of a material and of a device should be correlated with the time required by the healing process in which the material and device interact. After healing has been accomplished, the degradable implant is no longer needed and could even behave as an unwanted cause of a bone defect [2].

13.3.1 Cellular response to poly(lactic acid)

Synthetic bioresorbable polymers, such as PLA and its copolymers (PLA-based polymers), have attracted a lot of attention in the medical field. With their excellent biocompatibility, mechanical properties, and tunable biodegradability, PLA-based polymers have found uses in various clinical applications, including sutures and orthopedic fixation devices (e.g., pins, plates, and screws). PLA is widely used clinically as a biomedical scaffold for implants, theranostics, and drug delivery systems [225–227]. PLA-based polymers have also been the materials of choice for various cardiovascular applications [228]. The reason for the success of bioresorbable polymers in biomedical applications is twofold. First, bioresorbable polymers degrade *in situ* because of hydrolysis, thus avoiding the necessity of further medical operations for the removal of the device; the degradation products are metabolized by the organism itself. Second, a proper choice of design parameters allows for tuning of the drug release rate (e.g., when an active compound is loaded within the polymer matrix) or mechanical properties and their time evolution, because they are dynamically affected by degradation onset [229].

PLA is biocompatible, considered safe for direct contact with biological tissue, and is one of the few degradable materials approved by the US FDA and many other regulatory agencies [230]. Early study on PLA stent implanted in humans indicated its safe profile without inducing thrombosis and late stenosis for up to 6 months. However, further study on PLA reported that PLA may induce inflammatory response when implanted in the body due to their acidic degradation products, indicated by the presence of epithelioid and giant cells. Upon tissue implantation, the PLA polymer is coated by phagocytic cells and a fibrous capsule, denoting a foreign body reaction [231]. Also, were noticed macrophage cell damage, cell death and cell lysis upon phagocytosis of PLA particles [232]. The *inflammatory process* that accompanies the acute phase of the implantation of PLA-based materials has been studied in several *in vitro* models to better understand the roles of the cytokines and cell–material interactions. Hence, Nicolette et al. [233] have incubated PLGA microparticles with 2D J774 murine macrophage-like cells and observed an increase in both IL-1 β and TNF- α protein levels.

The degradation rate of PLA-based implants is dependent on pH and temperature in the tissue, on the one hand, and on the composition of the polymer, on the other. Therefore the degradation rate in sites of inflammation will be higher compared to healthy tissues [234]. *In vivo* degradation times for PLA depend on the application and circumstances. It typically varies from 50% in 1–2 years to 100% in 12–16 months, whereas PLGA (75:25) is typically fully degraded in 50–100 days [228,235]. Chirality also affects the degradation rate; D-PLA will degrade faster compared to L-PLA [234].

The primary mechanism by which PLA is degraded inside the body is hydrolysis of the ester-bond backbone and degradation occurs on the surface of the polymer and inside the polymer bulk, creating lactic acid monomers and oligomers [234]. The hydrolytic degradation is then further catalyzed by the newly formed carboxylic groups at the terminal ends of the cleaved PLA chains—Fig. 13.4 [234]. The hydrolysis may involve enzymatic

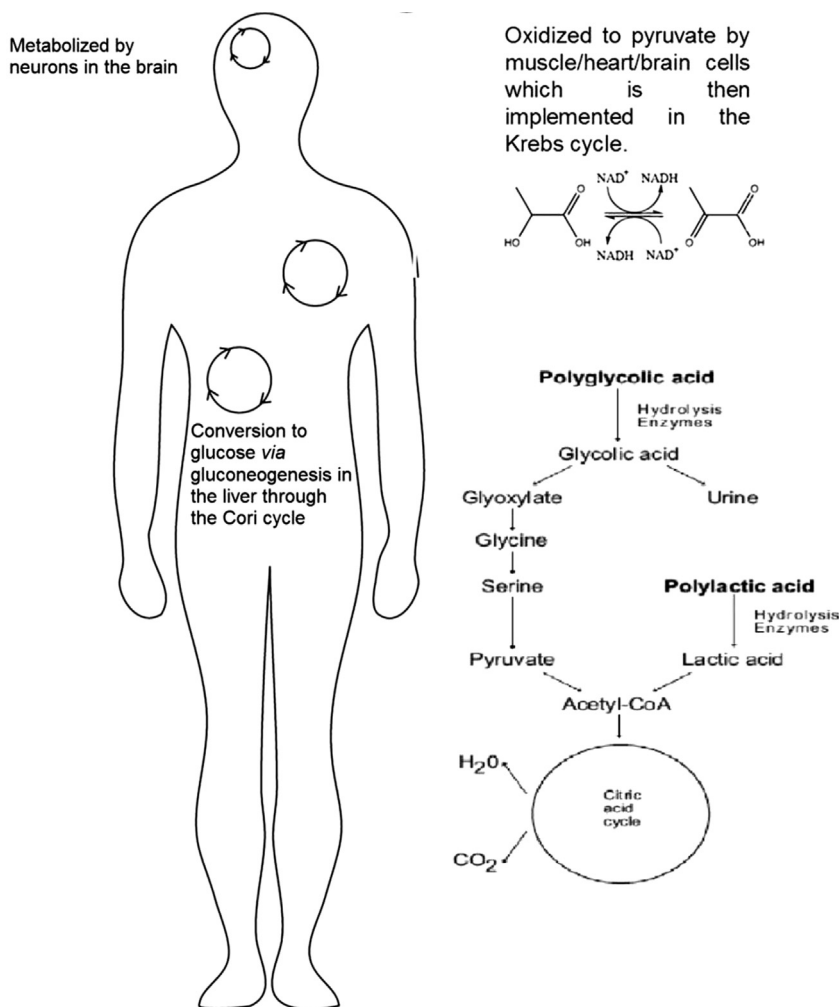


FIGURE 13.4 Natural biodegrading pathways of PLAPGA in primary sites of medical device implantation and principal occurring biodegradation reactions. PGA, Polyglycolic acid; PLA, polylactic acid. Source: Adapted from da Silva D, Kaduri M, Poley M, Adir O, Krinsky N, Shainsky-Roitman J, et al. Biocompatibility, biodegradation and excretion of polylactic acid (PLA) in medical implants and theranostic systems. *Chem Eng J* 2018;340(15):9–14; Peltoniemi H. Biocompatibility and fixation properties of absorbable miniplates and screws in growing calvarium. An experimental study in sheep [Academic dissertation]. Helsinki; 2000. Available from: <https://www.researchgate.net/publication/47934442_Biocompatibility_and_Fixation_Properties_of_Absorbable_Miniplates_and_Screws_in_Growing_Calvarium_An_experimental_study_in_sheep> [236].

action [235,237]. The lactic and glycolic acids formed are further metabolized to carbon dioxide and water in the Krebs cycle [238,239]. The acidic breakdown products of PLA can lead to a local decrease in pH of the tissue surrounding the implant, which may result in cell necrosis and inflammation [240].

A close look at radiolabeled PLA degradation products indicated that these are secreted from the body, and not retained in any primary organ [231]. It is assumed excretion occurs through kidney filtration and urine or as carbon dioxide [234].

The above mentioned bioresorbable polymers, however, do have disadvantages that may limit their applications as homopolymers, including slow degradation, poor mechanical ductility, and lack of specific biological interaction [241]. To overcome these limitations and tailor PLA properties to suit biomedical applications, PLA has been modified using different approaches such as surface modification, blending or composites [241–248].

13.3.2 Cellular response to polycarbonates

Among biodegradable synthetic polymers, aliphatic PCs have received much attention due to their excellent biocompatibility, biodegradability, and approval for biomedicine use by the FDA. Notably, PCs suffer from surface erosion degradation *in vivo* rather than bulk degradation in the hydrolysis of aliphatic polyesters [224,249]. Degradation of most PCs is controlled by the hydrolysis of the carbonate group which yields two alcohols and carbon dioxide thus alleviating the problem of acid bursting seen in polyesters, hence reducing the hazard of adverse reactions in bioapplications [250]. Since pure PCs degrade extremely slowly under physiological conditions, poly-iminocarbonates and tyrosine-based PCs have been engineered to yield biodegradable polymers of good mechanical strength for use in drug delivery and orthopedic applications. Structural variation of the pendant side groups allows for the preparation of polymers with different mechanical properties, degradation rates, as well as cellular response. PCs that contain a pendant ethyl ester group have been shown to be osteoconductive and to possess mechanical properties sufficient for load-bearing bone fixation. Long-term (48 weeks) *in vivo* degradation kinetics and host bone response to tyrosine-derived PCs were investigated using a canine bone chamber model [251]. Histological sections revealed intimate contact between bone and the tested PCs. It was concluded that, from a degradation–biocompatibility perspective, the tyrosine-derived PCs appear to be comparable, if not superior, to PLA in this model [250].

13.4 Conclusion and future trends

Biocompatible materials of plastic origin are considered a good way for an enhanced patient care, because they show small coefficient of friction. Manufacturers and researchers are working to improve its service life by developing more wear-resistant materials as the second-generation; HXLPE and antioxidant vitamin E within the PE are some examples [252]. New synthetic meshes are continuously developed, and new polymers and innovative coatings are continuously introduced as warp-knitted synthetic meshes composed of different polymers with different tensile properties, PEEK, polyamide, coatings as PP

mesh coated with PLLA exhibited an additional property of antiadhesion, electrospun nanofibers of various polymers as tissue scaffolds in hernia repair has been an active research topic in recent years [253].

There are some recent developments in the use of synthetic biomaterials as cellular substrates, including complexities associated with purification, immunogenicity, and pathogen transmission, a greater control over materials properties and tissue responses considering the synthetic analogs, creating sophisticated synthetic materials that interact with their biological environment so they participate actively in pathways of tissue morphogenesis.

Composites, hydrogels and nanostructures are extensively investigated to obtain improved surgical materials. Some of them constituted the topics of other chapters of this handbook.

Transitioning TCPS from a 2D substrate to a 3D one offers significant benefits. The potential exists to revolutionize cell culture [163]: 3D models have been shown to improve disease and pharmaceutical modeling [254,255] and capitalize on dynamic culture methods, generating clinically relevant geometries and numbers of cells [256,257].

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Cellular responses to zirconia

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14.1 Introduction

Zirconium dioxide (ZrO_2), as known as zirconia, is a bioceramic studied for the first time by the German chemist Martin Heinrich Klaproth in 1789 [1]. Its use as a biomaterial began in 1969 and concerned orthopedics application where ZrO_2 has been proposed as new material for hip head replacement instead of titanium or alumina prostheses [2]. It is a white and odorless crystalline oxide. Pure zirconia exists in three different crystalline structures: monoclinic (M) from room temperature up to 1170°C, tetragonal (T) from 1170°C to 2370°C, and cubic (C) over 2370°C (Fig. 14.1). The synthesizing temperature of pure zirconia is between 1500°C and 1700°C, and during cooling, there is the transformation from the tetragonal structure to the monoclinic structure (T–M) at about 1170°C with a volumetric expansion of about 3%–4%. This entails the establishment of residual stress with consequent cracks formation up to breakage [3]. The formation of cracks in the cooling phase is avoided by preventing the transformation of the tetragonal structure into a monoclinic structure thanks to the addition of refractory oxides grains such as CaO, MgO, Y_2O_3 , and CeO_2 ; these stabilize the cubic and tetragonal structure of zirconia at room temperature in a metastable state. If the quantities of the metastable tetragonal phase are sufficient, applying a stress on the surface there will be a force concentration at the tip of the crack which can induce the transition from the tetragonal to monoclinic phase, with the connected volume expansion (Fig. 14.2). This phase transformation puts the crack in compression, delaying its propagation and increasing fracture resistance.

In the early stages of development, many combinations were tested for biomedical application. However, in recent years research efforts have focused on the development of

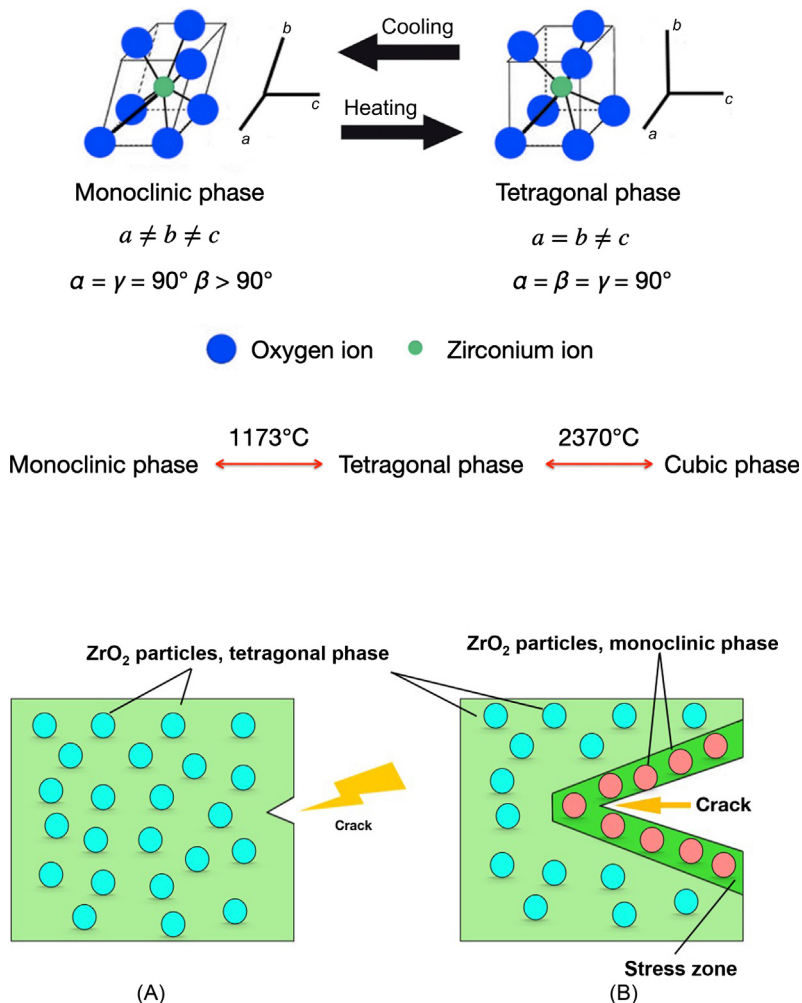


FIGURE 14.1 Scheme of the tetragonal and monoclinic crystal structures of zirconia and its changes of state depending on the temperature.

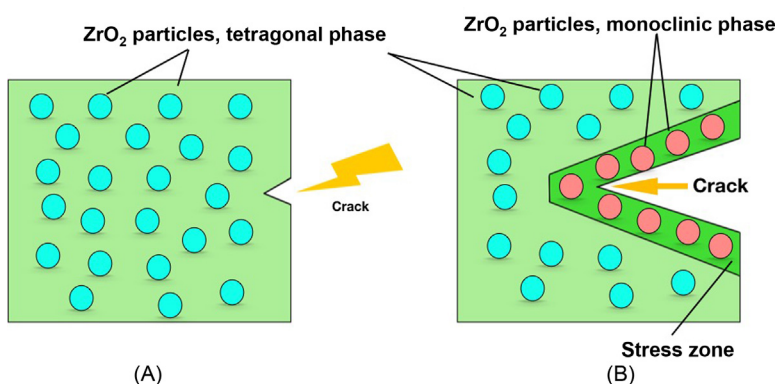


FIGURE 14.2 Schematic representation of the transformation of zirconia following a stress: (A) A crack before triggering the ZrO₂ phase transformation and (B) crack stop due to stresses from compression induced by the t-m transformation of zirconia.

zirconia-yttria ceramics, a combination commonly known as fully stabilized zirconia with yttria or yttria-tetragonal zirconia polycrystals (Y-TZP) [3]. The Y-TZP presents a higher fracture toughness among advanced ceramic materials [4]. Moreover, the Y-TZP has several characteristics such as high density, low porosity, high ductility, and compressive strength that favor use in the biomedical field. In the last 10 years the use of ceramics enriched with zirconia has increased significantly in biomedical applications, especially in orthopedics and dentistry, thanks to its mechanical characteristics such as: high hardness, resistance to erosion and fractures (breaking strength to straight bending of 900–1200 MPa), a elasticity module similar to that of the steel, a coefficient of thermal expansion similar to iron, a degree of hardness lower than that of alumina (1200 Vickers vs almost 1600) but still sufficiently valid.

Because of its mechanical properties, which are very similar to those of metals, the zirconia was called “ceramic steel” by Garvie in 1975 [5]. Compared to other ceramics, in addition, zirconia has a high biocompatibility. Together with its structural characteristics, this is one of the main reasons for the increase in the use of zirconia in the construction of prosthetic implants. The aim of this chapter is to analyze the response of cells to zirconia.

14.2 “Aging” of zirconia

Although most of the physicochemical characteristics of zirconia are much appreciated, much to prefer it to other ceramics, many works in the literature are devoted to the explanation of a less attractive feature of zirconia: its susceptibility to degradation at low temperature [6]. It is not possible to obtain any component with pure zirconium oxide, because its crystals modify their reticular structure according to the temperature spontaneously [7]. In an environment with high humidity, at temperatures between 150°C and 400°C, there is a slow but spontaneous transformation of the tetragonal phase of zirconia to the monoclinic phase. The transformation starts from the surface up to extend to the whole mass of the material and causes changes in the mechanical properties. The superficial tetragonal grains can spontaneously transform into monoclinics due to abrasive procedures, and this produces compressive stresses at the depth of several microns below the surface. The transformation of a grain is accompanied by a volumetric increase that causes stress on the surrounding environment with consequent formation of microcracking. The penetration of water then exacerbates the process of superficial degradation and the transformation propagates to neighboring grains. The progression of the tetragonal-monoclinic transformation can give rise to superficial cracks followed by detachment of the grains from the surface with deleterious effects on mechanical behavior, eventually producing the roughening of the surface. This phenomenon of degradation of zirconia is known as “aging” or low temperature degradation. The age of zirconia is directly related to the mechanical characteristics of the ceramic. The effects of aging are the reduction of strength, hardness, and density [8]. By adding the zirconia stabilizing oxides it is possible to move the stable sectors of the transformation phases from the melting point to the room temperature. This avoids the crystalline lattice of zirconia oxide being transformed, during cooling, from tetragonal to monoclinic. Various studies in the literature [9,10], in fact, report that the degradation is notable only for pure zirconia or zirconia with a low stabilizing content. It changes in the stabilized zirconia and depends on some parameters such as: concentration and distribution of the grains, the spatial gradient, but most of all the size of the grains. The more small the grains are, the more slow degradation is. Numerous studies have been conducted to measure zirconia aging kinetics. The laws that quantify the relationship between the transformation of the tetragonal and monoclinic phases with the time can be linear or sigmoidal. Up to now, no real effort to rationalize these differences has been made [11]. Despite the exact mechanism with which the degradation of zirconia occurs is still the subject of debate in literature [12], the production of waste from the surface could create problems in the use of the material in biological systems, compromising the characteristics of biocompatibility and favoring the host organism. Despite this, a few in the literature report cases of failure of zirconia implants are related to aging of the

material. However, today there is a clear lack of correlation between aging and clinical failures, this is due to the absence of rigorous scientific work that correlates aging and prosthetic failure. There is therefore a great need for more advanced studies on zirconia implants, in order to find a deeper correlation between the microstructure of the material and clinical results.

14.3 Definitions of biocompatibility, osseointegration, osteoinductivity, and osteoconductivity

Biocompatibility is a term composed of the prefix bio-(from the Greek βίος, “life, living being”) and the word compatibility, from the Latin cum patior (“participate in”). This translates to the expression “being in tune with”. The concept of biocompatibility is therefore based on the part on the capacity of the material used not to provoke adverse reactions in the host and on the other hand in the host’s ability not to seek the destruction or expulsion of the aforementioned material. The biomaterial itself and its degradation products must not be responsible for inflammatory, allergic, autoimmune, toxic, mutagenic, or carcinogenic reactions. The term osseointegration, used for the first time by Swedish biotechnologist Per-Ingvar Brånemark, indicates the intimate union between a bone and an artificial implant without the formation of connective tissue between the two surfaces; the union is defined as intimate when the space and the relative movements between bone and implant do not exceed 100 μm . The speed of the osseointegration process and its quantity vary according to the type of implant surface [13]. Osseointegration is a time-dependent healing process in which the rigid fixation of alloplastic materials is obtained and maintained in the bone during functional loading [14]. It depends on the ability of the host’s cells to anchor on the surface of the biomaterial.

Fixation of alloplastic materials is obtained and maintained in the bone during functional loading [14]. It depends on the ability of the host’s cells to anchor on the surface of the biomaterial. Bone is a very dynamic tissue, during its lifetime it undergoes multiple modifications and remodeling phenomena to respond to harmful and nonstimuli [15]. Thanks to this ability, the bone is able to repair small damage and regenerate itself. An ideal biomaterial should be able to guide this repair process [16]. It should be able to drag the osteoprogenitor cells into the lesion and stimulate their growth and differentiation. This phenomenon is called osteoinduction. Osteoconductivity, on the other hand, is the ability of the biomaterial to grow the bone on its surface and to make the bone conform to it.

In the case of zirconium, the connective tissue reaction of the organism against it is negligible, as is the release of residues and other degradation products. Zirconia in fact produces a tissue inflammation reaction lower than that of the other ceramics, although it does not invalidate the osseointegration process and facilitate the osteoconductivity. In effect it seems that the material has the property of self-regulating the turnover of the extracellular matrix by acting on the expression of the related cellular genes [17]. The reaction of bone, connective tissue and blood is involved in the response of the host to ceramic implants. The study of reactions at the material–tissue interface of the host

should be conducted in vivo and in vitro to correctly evaluate the biocompatibility of an inert ceramic.

14.4 In vitro zirconia biocompatibility

In vitro tests use zirconia in different physical forms (disks, powders, sticks) and study the response of one or more cell lines in culture. Most of the in vitro tests performed did not demonstrate any type of acute toxic responses with the different cell lines tested [18]. In vitro tests are influenced by the chemical-physical characteristics of the material, for example, the shape, the impurity content, and the amplitude of the reactive surface and the cell conditions during the tests. In order to evaluate the basal cytotoxicity of nanoparticles, numerous in vitro, colorimetric and non, in vitro techniques can be used. Among the first widely used is, for example, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which exploits the ability of mitochondrial dehydrogenases to metabolize tetrazolium salts, releasing a colored compound, as an index of cell viability. Because of their chemical and physical properties, the nanoparticles can interfere with the reagents used in colorimetric assays producing results falsified by such interference [19]. Therefore a valid alternative is the use of noncolorimetric tests, such as the colony formation efficiency (CFE) assay, as it allows to evaluate cell viability as the ability of a single cell to form a colony, without the use of coloring or fluorescent substances. It also appears to be even more sensitive than conventional biochemical methods, because it does not measure a specific biological effect but rather cell death in general [20].

14.4.1 Cellular response of the fibroblasts

The most analyzed organic structure in vitro is represented by the connective tissue, being it is the most widespread in the human organism and the most involved in the healing processes. It is composed of a matrix composed of glycosaminoglycans, collagen fibers, water, and a cellular component, formed by fibroblasts and fibrocytes [20] (Fig. 14.3).

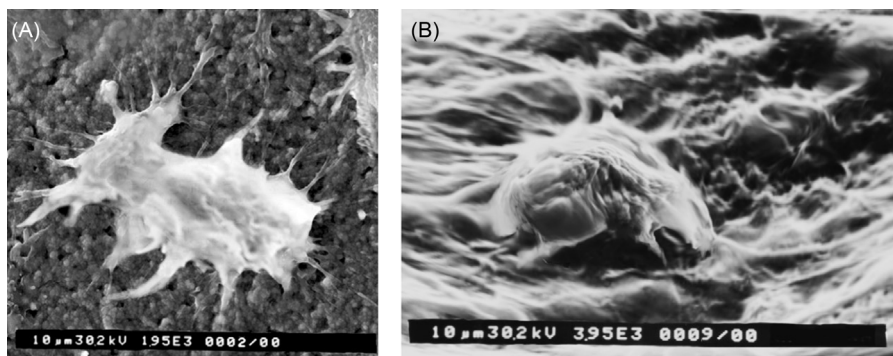


FIGURE 14.3 SEM fibroblasts in culture on a zirconia surface: (A) cells cultured on zirconia ceramic for 2 days and (B) after 6 days the cells cover the entire zirconium surface. A fibroblast body is evident. Power magnification: (A) $\times 1950$ and (B) $\times 3950$. SEM, Scanning electron microscopy.

Several authors have studied the cytotoxic effects of zirconia on fibroblasts. Kwon and Ito [21,22] compared the cytotoxic effects obtained from titanium oxide powder and zirconium oxide powder which may represent the normal product of material wear. While zirconia powder caused cell growth inhibition, titanium oxide powder did not cause any inhibition. Furthermore, the cytotoxic effect appears to be higher for particles smaller than 22 μm . Li et al. [23] evaluated cytotoxicity, on human fibroblast colonies, tricalcium phosphate powders, hydroxyapatite, and zirconium oxide. Cell viability was assessed using the MTT and CFE assays, demonstrating a 50% reduction in CFE at a concentration of 50 $\mu\text{g/mL}$ for zirconium oxide and no cytotoxicity for the other two materials used. Atalay et al. [24] tested three different concentrations (50, 100, and 150 $\mu\text{g/mL}$) of 20 nm-sized zirconium nanoparticles in fibroblast cultures demonstrating a cytotoxic and genotoxic effect at all the concentrations used. The most cytotoxic form, however, seems to be that in powder form. Harmand et al. [25] demonstrated instead the absence of cytotoxicity, by using the MTT test for zirconia in cultures of human fibroblasts. Others have even shown that zirconia has a greater superficial cell adhesion than titanium [26]. Yamano et al. [27] in a recent study evaluated the proliferation of fibroblastic cell lines on the zirconia and titanium surfaces, demonstrating that on smooth zirconia, cells proliferated much faster than titanium. In addition, the $\alpha 2$ and $\alpha 5$ integrin gene expression of type I collagen is much greater in zirconia than in titanium in the first 48 hours, whereas integrin $\beta 1$, $\beta 3$, and collagen type III expression are significantly lower than in other materials. This demonstrates that bio-material can stimulate and alter the response of host cells.

14.4.2 Cellular response of leukocyte cell lines

Monocytes, lymphocytes, macrophages, and other cells of the immune system are a very important part of the hematological elements contained in the connective tissue. The analysis of their behavior represents a fundamental step in the study of the levels of biocompatibility of the zirconium. Using zirconium powders on human lymphocytes, some authors evaluated mitotic levels after stimulation with hemagglutinin and observed the presence of cytotoxicity directly proportional to the concentration of the powders used [28]. Mebouta-Nkamgeu et al. [29] have shown that by adding zirconia powder to macrophage cultures at 37°C in a 5% humidified CO₂ atmosphere for 7 days, there is a reduction in macrophage cell activity, a reduction in the potency of phagocytosis, and control of oxidative burst as well as a cytotoxicity directly proportional to the concentration of powder used, the mortality of macrophages increases as the size and concentration of particles larger than 2 μm increases. However zirconia proved to be less toxic than alumina. Catelas et al. [30] studied the apoptotic effect of various high-density ceramics and polyethylene (HDP) on murine macrophage culture lines. The effect of ceramic particles on the induction of apoptosis was dependent on their size and concentration. This effect reached a plateau for more than 150 macrophage particles from all the compounds examined (Al₂O₃ vs ZrO₂ and Al₂O₃ vs HDP). In addition to the evaluation of cytotoxicity on immune cells, it is also important to evaluate the ability of ceramics powders to produce an inflammatory response. In fact, several authors have studied the production of inflammatory mediators in macrophage cell lines incubated with ceramic and metal materials. Sterner et al. [31]

studied the $\text{TNF}\alpha$ response of macrophage-like cells (THP-1 cells, monocytic human cell line) after exposure to ceramic powders. Alumina ceramic (Al_2O_3), ceramic zirconia (ZrO_2), and titanium (Ti) particles of different sizes and concentrations have been considered. Titanium particles caused the largest response of $\text{TNF}\alpha$, which also seemed to be related to particle size. Al_2O_3 particles are not as effective, but four times more $\text{TNF}\alpha$ have been released than control. ZrO_2 showed no effects on the release of $\text{TNF}\alpha$. Warashima et al. [32] reported less proinflammatory mediators (IL-1-b, IL-6, and $\text{TNF}\alpha$ generated by ZrO_2 than titanium or polyethylene).

However, this does not explain the results obtained by Bu et al. [33]. Among the cytokines released during the interaction between the ceramic particles, including zirconia, and macrophage cell matrices, the interferon- γ (IFN- γ) resulted to be a critical functional factor for osteoclast differentiation and which can guide the osseointegration process. Zirconia powders inhibit the secretion of IFN- γ in human monocytes. This would appear to be caused by an increase in $\text{TNF}\alpha$. Obando-Pereda et al. [34] demonstrated that macrophages in culture with zirconium or titanium oxide particles expressed an increase in mRNA levels for TLR 2, 3, 4, and 9 and their MyD88, TIR-domain-containing adapter-inducing interferon- β (TRIF) and NF- κ B adapters and cytokines $\text{TNF}\alpha$, IL-1 β , and IL-6. Quantitative differences are evident and, in general, the proinflammatory gene expression induced by zirconium oxide particles was lower than that induced by titanium particles.

Some authors also argue that the surface structure that interacts with the cell substrate is more important than the chemical composition of the material. In fact, Wang et al. [35] have shown that hydrophilic surfaces tend to reduce the proinflammatory response by lowering the gene expression of $\text{TNF}\alpha$, IL-1, and IL-6 and promoting tissue resolution through the expression of a cytokine IL-10. It also seems that the topography and chemistry of the implant surface have a substantial impact on the behavior of macrophages. In fact the presence of superficial hydrophilicity would improve the integration of the implant in the soft tissues despite the chemical composition of the material [36].

14.4.3 Cellular response of osteoblasts and osteoclast

Many authors have evaluated the response of osteoblasts and osteoclasts in culture to powders and other forms of ceramic materials. The biocompatibility tests on osteoblasts are in fact important for the study of implant osseointegration, this is functional to the best clinical outcome obtainable. It has been observed that zirconia has no cytotoxic effects and is able to interact with the osteoblasts that are adjacent to it, allowing cells to form the extracellular matrix through the synthesis of different and essential structural proteins. Zirconium does not appear to induce any teratogenic adverse effect by not making changes to DNA [37]. Torricelli et al. [38] studied the response of murine osteoblasts to a ceramic (RKKP)-coated with zirconium oxide or aluminum oxide. Cell proliferation (MTT test) and cell differentiation (alkaline phosphatase activity) were measured at the end of the experiment to evaluate the behavior of osteoblasts in the presence of biomaterials. The results of both materials showed a good level of biocompatibility. In particular, significant higher MTT values were detected on ZrO_2 -RKKP samples. Lohmann et al. [39] analyzed the proliferation of MG-63 osteoblastic cell lines in contact with alumina and zirconium

particles, demonstrating greater cell proliferation in the zirconia contact samples than those in contact with alumina. Other authors have instead observed how the presence of zirconium impurities can influence molecular level on osteoclasts and favor the possible osteolysis through a cell activation of macrophages and a degradation of the particles by osteoclasts thanks to the production of oxygen-free radicals [40]. In recent years, the ability of biomaterials, including zirconia, to influence gene expression and cell differentiation has become increasingly important. Da Costa Fernandes et al. [41] argue, in a recent study, that zirconia triggers important intracellular signals that lead to changes in cell adhesion and proliferation through changes in the intracellular pathway signals. In fact it seems that the phosphorylation of focal adhesion kinase and Rac1 are decreased in cell cultures of osteoblasts treated with zirconia powder. This would result in a reduction in cell adhesion and a stimulus to proliferation. Zirconia would also act on the organization of the cellular cytoskeleton and on the remodeling of the extracellular matrix. Altmann et al. [42] conducted a detailed analysis of the gene modulation that occurred in cell cultures of osteoblasts incubated with zirconia. It has shown a dual dependence of transcriptional changes from biomaterial chemistry and surface topography. Among the genes most stimulated by zirconia we recognize various osteogenic bone morphogenetic proteins (BMPs) and transcription factors, matrix collagen, osteocalcin, laminin, β 1 integrin, and MMP-2. This would explain the biocompatibility of zirconia and its ability to stimulate remodeling of the extracellular matrix and stimulate osseointegration. Furthermore, several authors have carried out an accurate analysis on the possibility of carcinogenicity of zirconia without finding significant data [43].

14.5 In vivo zirconia biocompatibility

Although the use of in vitro tests is fundamental in the study of biocompatibility of zirconia, the study of complex physiological phenomena related to organs or systems in their entirety requires the use of appropriate models. This is the reason why in vitro tests should be supported by in vivo tests. The first experimental data of zirconia in vivo biocompatibility were published in 1969 by Helmer and Driskell who have studied zirconia balls implanted in monkey's femurs. No adverse effects or toxicity were found [44]. Since then, different animal models have therefore been used for the evaluation of biological reactions to zirconia in the form of solid masses, particles, fibers, and coatings. Christel et al. [45] studied the reaction occurring in rats after the implantation of zirconia and alumina polycrystals in the paravertebral muscles. The materials studied through histomorphometric techniques gave a similar biological response, in fact the polycrystals of zirconia and alumina implanted for more than 12 weeks were encapsulated by fibrous membranes of similar consistency and thickness. Garvie et al. [46] studied in vivo the magnesium stabilized zirconia response in paraspinal muscles of rabbits. At 6 months from the inoculum there was no adverse effect on the soft tissue and no wear of the implanted material occurred. Several authors claim that angiogenesis and in particular the vascular endothelial growth factor appear to be extremely important for tissue preservation, but also for the development of periimplant inflammatory reactions and in soft tissues [47]. These reactions were studied by Degidi et al., which showed higher responses in titanium

compared to zirconia [48]. Zirconium seems to interact actively with soft tissues by activating different cell pathways that allow the growth of fibrous connective tissue at the implant interface which is gradually transformed into bone tissue, as demonstrated by biopsy findings of periimplant tissues in patients in whom titanium screws coated with zirconium are implanted [47–49]. Maccauro et al. [50] studied in vivo the response to the implantation of zirconia toughened alumina cylinders implanted in cavities created surgically in both rabbits tibiae. No sign of osteorarfaction was detected at the radiographic study 12 months after implantation; no tumor developed after implantation either locally or in other organs, which testifies to the high biocompatibility of the material [51]. Hamad et al. [52] studied the response to coated and uncoated zirconia titanium screws. Mechanically, nano-zirconia-coated implants showed a statistically significant difference in removal torque values, whereas histologically these coated implants improved and promoted osseointegration after 4 and 12 weeks of healing, compared to uncoated ones. Chung et al. [53] evaluated osseointegration in rabbit tibias of zirconia-coated implants (smooth or rough) and uncoated implants. Zirconium-coated implants, both smooth and rough, showed a better histological response (bone-to-implant contact) than uncoated ones. On the other hand, the mechanical anchorage was higher for plants with a rough surface. Another important aspect to be analyzed regarding the biocompatibility of zirconium towards soft tissues is related to tribological aspects and to the ability of degradation products or powders to induce or not responses in the host. In this regard, no local or systemic reactions were observed after the peritoneal implantation of partially stabilized zirconium powder containing calcium or yttrium in mice [54]. Zirconium, in whatever physical form is tested, does not appear to induce cytotoxicity in the soft tissues even if the levels of immune system cells in the periimplant region increase.

14.6 Conclusion

In the various analyzation papers emerges the greater ability of zirconia compared to other materials, including titanium and alumina, to stimulate cell proliferation, also through gene expression modifications. An important role is played by the conformation of the reaction surface. This is the reason why researchers' efforts should be aimed to finding a material that can guarantee maximum cell proliferation accompanied by optimal adhesion in order to achieve greater osseointegration. In fact, in recent years technological progress has allowed the development of new types of stabilized zirconia; with modern processing techniques it is possible, through surface modeling, to produce increasingly pure and biocompatible materials; the consequence is the overcoming of many limits related to the use of alumina. Studies published to date in the literature show that zirconia has excellent mechanical, biological, and tribological characteristics. The lack of carcinogenicity and local and systemic toxicity combined with the strength of the material and its ability to promote osseointegration make this material an ideal coating for prosthetic implants. Although in the past zirconia has been widely used in the orthopedics, the long-term results have been questionable, so much so that its use has recently come to a halt. An area in which the use of zirconia is increasingly frequent is dentistry where, for esthetic reasons, the mechanical and biological properties, zirconia's mechanical and

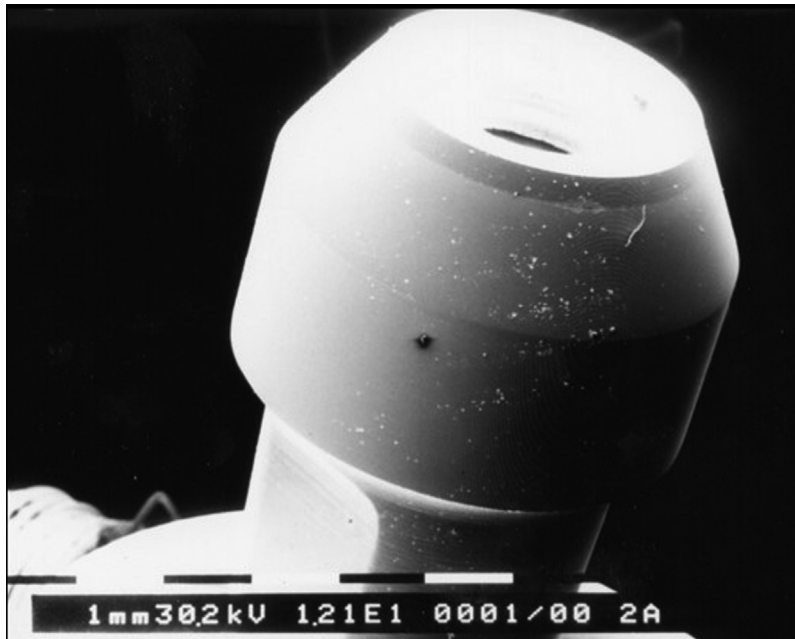


FIGURE 14.4 Use of zirconia in dentistry—replacement missing teeth with an implant supported restoration: SEM image of a zirconia CAD–CAM abutment for implant supported restoration. Power magnification: $\times 1210$. CAD, Computer-aided design; CAM, Computer-aided manufacturing; SEM, Scanning electron microscopy.

biological properties are ideal materials for the construction of dental implants (Fig. 14.4). The aging of zirconia can have negative effects on its mechanical properties. This phenomenon in the clinical setting is not yet fully understood. While in orthopedics this feature can be a limiting factor, in dentistry the reduction of mechanical resistance does not seem to affect clinical outcomes. However, further studies are needed to guide the biomedical use of zirconia with regard to bonding with other ceramics, anchoring procedures, and material aging.

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Cellular response to alumina

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15.1 Introduction

Interaction of biomaterial surface with different mechanisms of normal healing process plays a vital role in success or failure of tissue integration. The effects of the implant on the host tissue and the effects of the host on the implant are crucial problems of medical devices. Many researches have paid attention to the development of materials and coatings with improved tissue compatibility which lead to the creation of several new biomaterials. Unluckily, some of these materials still can trigger host inflammatory responses, which in some conditions cause failure of the implanted device [1–4].

When a biomaterial is implanted into the body, several immunological reactions hierarchically happened: wound, blood and material interactions, acute and chronic inflammation, granulation tissue formation, foreign body responses, and eventually formation of a fibrous capsule [5–9]. It has been shown that tissue destruction could be caused by reduction of foreign body responses, such as infiltration of macrophages. Therefore carefully considering the details of immune system responses to the implanted material is essential in designing a biomaterial [10]. In initial interactions between the cells and biomaterials the adsorbed proteins, also macrophages and dendritic cells, have key roles when the biomaterials are implanted in the body [11–13]. It has been known that the quick adsorption of proteins on the surface of the biomaterial depends on the physicochemical properties of both biomaterials' surface and proteins [14]. In recent decades, because of their excellent biocompatibility and biomechanical properties, ceramics gained attention in researches

and clinical manufactures. According to in vivo behavior, bioceramics are categorized as bioresorbable, bioactive, and bioinert [15].

Bioinert ceramics such as alumina and zirconia are used in many orthopedic devices [16]. It has been shown, due to their properties such as low wear and high stability, they attained their most popular application in arthroprosthetic joints, although, these ceramics will be more effective in younger, active patients [17].

In the first use of alumina, called aluminum oxide, Al_2O_3 , Rock applied for a patent in 1930. Then, in 1965, Sandhaus designed a dental implant made of the alumina powder that opened the era of advanced alumina ceramics [18,19].

However, the first used alumina in orthopedic surgery was performed by Boutin in 1970 when he implanted alumina joints [20]. More than 3 million alumina ball heads have been implanted worldwide, in the clinical usage of alumina for more than 30 years [21,22]. Today, alumina has been developed in more purity, full density, and enhanced micro-structure. The average grain size of alumina is reduced whereas bending strength in clinical-grade has increased [22]. Because the use of alumina has expanded in dental and orthopedic biomaterial applications, studying the biocompatibility of it has an importance, which is the main focus of this chapter.

In the present chapter, alumina's surface critical physicochemical properties which affect cells and proteins adhesion will be discussed thoroughly.

15.2 Physicochemical properties of alumina surface

The investigations have shown that the physicochemical properties of ceramic biomaterials are very important in expanding their use of them in biomedical applications [23,24]. Alumina is a ceramic with high hardness and abrasion resistance properties. The exceptional wear and friction properties of alumina, are the results of surface energy and the smoothness of it [18,22]. Because of various useful properties of Al_2O_3 , it has a variety of uses in biomedical devices. In Table 15.1, many properties of alumina and some common oxides are summarized [25,26]. These oxides can be in alumina as contaminants or additives. $\alpha\text{-Al}_2\text{O}_3$ (corundum) with hexagonal structure is the stable crystalline phase of alumina [26,27]. In alumina structure, oxygen anions are packed into a hexagonal sublattice and small-sized aluminum cations have filled two-thirds of the octahedral interstitial centers of the oxygen sublattice [27,28]. Cubic $\gamma\text{-Al}_2\text{O}_3$ is one of the metastable crystalline phases of alumina that is formed by crystallization of aqueous solutions or dehydration of aluminum hydroxides. The metastable phases of alumina can convert to $\alpha\text{-Al}_2\text{O}_3$ in temperatures above 1000°C , and temperatures of up to 1450°C may be needed for completing recrystallization. The metastable phases of Al_2O_3 have restricted applications, for example, sorbents, catalyst vehicles, and raw materials to form α -alumina [29–31].

Some studies have shown that when the surfaces of a metal oxide such as alumina are exposed to water or air, $-\text{OH}$ groups would start to form on the surface, which later helps them in obtaining more communications with the adsorbed proteins. The high difference in elasticity between the biological tissue and the alumina-based biomaterials is the result of the stiffness of alumina [32]. Today, due to great mechanical properties for dentistry and orthopedic applications, alumina-based biomaterials have achieved high attention among

TABLE 15.1 Alumina, zirconia, silica, titania, and magnesia's properties.

Property	$\alpha\text{-Al}_2\text{O}_3$	SiO_2	ZrO_2	TiO_2	MgO	Reference
Density (g/cm^3)	3.99	2.20–2.65	(5.80–6.05) ^a	4	3.58	[27]
	3–3.98	2.20 ^b , 2.65 ^c	5–6.15	4.24	3.54–3.58	[26]
Melting point (T_m) ($^{\circ}\text{C}$)	2054	1710 ^d	2710	1855	2852	[27]
	2004–96	–	2550–2700	–	2807–62	[26]
Ultimate strength, MPa						
Flexure	282	310	430–720 ^a	340	441	[27]
	152–800	110–200	177–1000	140	100–200	[26]
Elastic modulus (E) (GPa)	365–393	73	200 ^a	248–282	303	[27]
	215–413	66–75	100–250	230	270–330	[26]
Resistance of crack (K_{1c}) ($\text{MPa} \cdot \text{m}^{0.5}$)	–	–	(7–15) ^a	–	–	[27]
	3.3–5.0	0.62–0.67	1–8	3.2	2.7–2.8	[26]
Hardness						
Vickers (HV) (GPa)	20.6–29.4 5.5–22.0	8 4.5–9.5	14.4, 15.7 5.5–15.8	– 8.6	7.4 5–7	[27] [26]
Mohs (HM)	9 9	7 7	6.5 ^e 8	7.0–7.5 6.5	5.5–6.0 –	[27] [26]

^aCubic partially stabilized MgO (~ 3 wt.%).^bQuartz glass.^cQuartz ($\alpha\text{-SiO}_2$).^dCristobalite.^eBaddeleyite.

biomedical specialists. Also, an important factor that can be helpful in orthopedic applications is bending strength [33]. The mechanical properties of alumina can be adjusted by its processing principles such as sintering, quality control, and powder processing. Currently, alumina is still known as a fragile material, because yielding at the crack tip could not consume fracture energy, thus alumina could not show any flexibilities [34,35]. Therefore the mechanical properties of alumina principally depend on the presence of defects [35]. Some researchers have shown that the bending properties of alumina have a reverse nonlinear dependence on porosity. Moreover, the amount and interconnectivity of pores are two additional important factors that have an impact on the fatigue properties of alumina [36]. It has been shown that interconnect porosity fraction has an individually high influence on mechanical properties of alumina-based biomaterials for long-term in body fluids [37,38]. Also, subcritical crack extension by water molecules is another important factor leading to mechanical strength of alumina reduction [39]. Introducing high-purity powders that contain smaller amounts of additives such as calcium oxide, alkali, and silica can solve the problem of strength degradation in alumina-based biomaterials. These additives play a vital

role in alumina-based biomaterials' mechanical stability [36]. Some studies demonstrated that calcium oxide interacts with water molecules and leads to strength of alumina-based biomaterials reduction, while silica prevents densification and promotes grain growth during sintering process, which increases fatigue fracture. Also, silica and alkalis can be located at grain boundaries, which increase the fatigue fracture by the dissolution of these in body fluids [40,41]. Recently, to improve the mechanical properties of alumina-based biomaterials, various formulations and processing methods have been developed. These are based on two fundamental strategies including flaw reduction strategy and microstructural purification [36].

15.3 Cellular responses and protein adsorption on alumina surface

Many studies have been performed on the interactions between alumina and cells and biomolecules in the body [42–45]. Nevertheless, alumina has obtained a prominent position in skeletal regeneration; the investigations on biocompatibility and cellular responses continue. Several cell lines such as osteoblasts and fibroblasts have been applied to in vitro biocompatibility assay and immunological cells with different cell environments [44,46]. There are two approaches in the examination of the alumina cellular responses including contact with the cells in the culture media in a direct or indirect manner. The investigations have shown that using the indirect approach, important biological reactivity might be ignored. Although the results of the direct cell contact method showed that alumina powders' concentration could determine the amount of toxicity [36]. Also, responses of cells and different molecules can be changed by different physical forms of alumina, for example, powders, granules, and porous or dense structures [47]. It has been reported that during in vivo tests, the change of the physical features of a biomaterial can cause changes in biological responses to it; which with alumina, due to resealing the wear particles into the body, this fact has major importance. So, it is consequent that the alumina nanoparticles, more than monolithic alumina, cause inflammatory responses and aseptic loosening [48,49]. The vitality of surface contact areas in cellular responses to the surface of biomaterials is explained in many studies. Also, it has been shown that biocompatibility can be affected by the size of alumina particles [43,50,51]. Nevertheless, the relation between cellular responses and particle dimension of alumina, has not been clarified. Ferraz et al. [43], in their research, have shown that alumina nanotopography could change the behavior of monocyte and macrophage. In this study, human mononuclear cells cultured on alumina membranes with pore between 20 and 200 nm. Then they have assessed cell adhesion, viability of cells, morphology, and the release of proinflammatory cytokines. In this research, they have evaluated cell adhesion by light microscopy, viability of cells by evaluating lactate dehydrogenase release, and the inflammatory reactions by measuring interleukin-1 β and tumor necrosis factor- α . This study showed that the cell number, morphology, and cytokine release can be changed by the nanoporosity. Also, on the porous alumina surface with pore size 200 nm few cells with high activities were found, while the porous alumina surface with pore size 20 nm was observed with a greater quantity of cells. Nonetheless, despite that the greater quantity of cells adhered to the 20 nm surface, proinflammatory activities have been reduced. Finally, this study showed that nanotopography could be employed to manage the inflammatory reactions to the surface of implantable biomaterials

[43]. According to some papers, two main mechanisms can play vital roles in macrophages' response to the topography of biomaterials' surface: first, nanotopography of the surface could affect the adsorption of proteins [52]. Second, surface topography could have effects on proliferation, adhesion, and also cells differentiation [53]. Previous studies have determined that nanostructures can be recognized by cells, which results in altering cell phenotype and functions [54–56]. In another study by Ferazz et al. [57], it was shown that the adsorbed plasma proteins which contain IgM, IgG, C3, and C1q on alumina surfaces with 200 nm pore size were more than the 20 nm. Therefore the alumina surfaces with larger pore sizes are more complement system activating [57].

In addition, because the knowledge of osteoblast response to the topography of biomaterials surface is essential for effective bone tissue engineering applications, therefore, some research has been performed on evaluating the osteoblasts cell responses on nanoporous surface of alumina [42,58]. In their research, Mussano et al. [59] used two AlO surfaces with different pore sizes in the mean range of 16–30 and 65–89 nm. Then the two alumina surfaces were characterized by field emission scanning electron microscope and energy dispersive x-ray spectroscopy. In vitro cell response including initial cell adhesion, viability, morphology, and focal adhesion quantification were evaluated. The results showed that both alumina surfaces can increase the cell adhesion and viability more than the control surface. Osteogenic differentiation showed that the nanosurfaces with 65–89 nm pore size are more efficient than 16–30 nm; this data was evaluated by alkaline phosphatase (ALP) activity and extracellular calcium deposition. The results of the comparison of two nanoporous alumina surfaces with various pore sizes confirmed the impact of surfaces' nanotopography in making a cell response [59].

Also, in another study, Leary Swan et al. [60] used a two-step anodization process to optimize the fabrication of nanoporous alumina membranes with uniform pore size and distribution. They claimed that anodizing voltage can affect the forming of nanoporous alumina membranes with pore sizes ranging from 30 to 80 nm in size. In this study the influence of the nanoscale pores on osteoblast response was analyzed. The in vitro result showed that the cells were spreading processes into the nanopores. These results proved that the osteoblasts responded to the nanoarchitecture. Also, they found that cells at different steps of culture revealed osteoblasts showing a normal phenotype and morphology [60].

Despite the fact that alumina is amongst the most conventional implant materials used in orthopedics, there is little data presented on the cellular responses of mesenchymal stem cells (MSCs) on nanoporous alumina. The responses of MSCs on alumina scaffolds with the smooth and nanoporous surface were examined in Song et al.'s research [61]. In their study, the pore sizes of alumina scaffolds were 20 and 100 nm and the impacts on cell responses were evaluated. These responses included proliferation, morphology, expression of integrin $\beta 1$, and osteogenic differentiation. In this study, by increasing the porous size of alumina scaffolds, cell viability decreased. On the alumina scaffold with the larger pore size, very elongated cells and prominent cell membrane protrusions were observed. Moreover, the integrin $\beta 1$ expression in MSCs cultured on porous alumina was increased, and this data can show that the porous scaffolds are more suitable than the smooth scaffolds. Between alumina porous scaffolds, the alumina scaffold with 100 nm pores exhibited upper concentrations of osteoblastic differentiation markers in MSCs

cultured. Overall, this work proved that cell responses to biomaterials are extremely reliant on their porous sizes and pore structures [61]. The same author in another research has studied the specific MG63 responses to nanoporous alumina surfaces. Variations of cell morphology, cell viability, expression of integrin $\beta 1$, and ALP activity were analyzed. The results exhibited that the increase in the pore size of alumina decreased the expression of integrin $\beta 1$ and cell viability, and cell morphology enlarged, and the ALP activity and mineralization increased. This study showed that the surface topography of nanoporous alumina plays a vital role in improving the MG63 osteoblast-like cells behaviors and consequently porous alumina can be considered as a helpful substrate in tissue engineering (see Fig. 15.1) [62].

Some studies showed that epithelial cells might be more compatible with culture on alumina surfaces in the 30 nm size in comparison with larger pore sizes [63]. The biocompatibility of glass slides and silicon nanoparticles with different sizes 20 and 310 nm has been examined in a study where alumina was used to coat the nanoparticles by employing atomic layer deposition. The results showed osteoblasts and human dermal fibroblasts have good proliferation on nanoparticles' surfaces with alumina coating. Moreover, only when the macrophages and nanoparticles at 1000 $\mu\text{g/mL}$ concentration have interactions, reactive oxygen species (ROS) are released by macrophages. A higher release of ROS were found in the nanoparticles with 20 nm in size rather than the 310 nm nanoparticles. Finally, this study exhibited alumina coatings that do not stimulate immunological cells [64].

Inflammatory response with the neutrophil, is one of the key factors in determining the biocompatibility of an implant, while it is one of the first cell types to attack the implanted substance [65–67]. One study evaluates neutrophil responses to alumina membranes by in vitro cell culture. This study revealed that the morphology and activation of leukocyte significantly depend on the pore size of alumina membranes (20 and 200 nm in size). However, the results have shown that spreading and extending pseudopodia of polymorphonuclear granulocytes was more observed on the 20-nm pore-size membranes than the 200-nm pore-size membranes. Also, adherent neutrophils on alumina membranes with the 20-nm pore-size product are much stronger primary oxygen free radicals. Finally, this research has shown that the pore size of the membrane significantly influences the extent of cellular responses of adherent neutrophils [68].

Quickly following implantation, the biomaterial will have interaction with blood. So the plasma proteins will immediately cover the implant surfaces. Since the adsorption of proteins on the biomaterial surface is performed faster than the migration of cells to a biomaterial surface, this produced film has high importance for the adsorption and activation of phagocytes [69–71]. Many types of research have shown that materials with precoated protein films on the surface will cause different neutrophils responses [72–74]. According to this fact, Karlsson et al. [68] evaluated human neutrophil responses to alumina membrane using different pore sizes, 20 and 200 nm, that were both precoated and uncoated with serum, collagen I, or fibrinogen. This study has also evaluated how the viability of osteoblastic cells (MG63) attached to the alumina membranes have influenced from released neutrophil granule components. The results have shown that the uncoated membranes with 200 nm pore-size have lower ROS than the alumina membranes with 20 nm pore-size. These pore-size depending responses were also observed on membranes

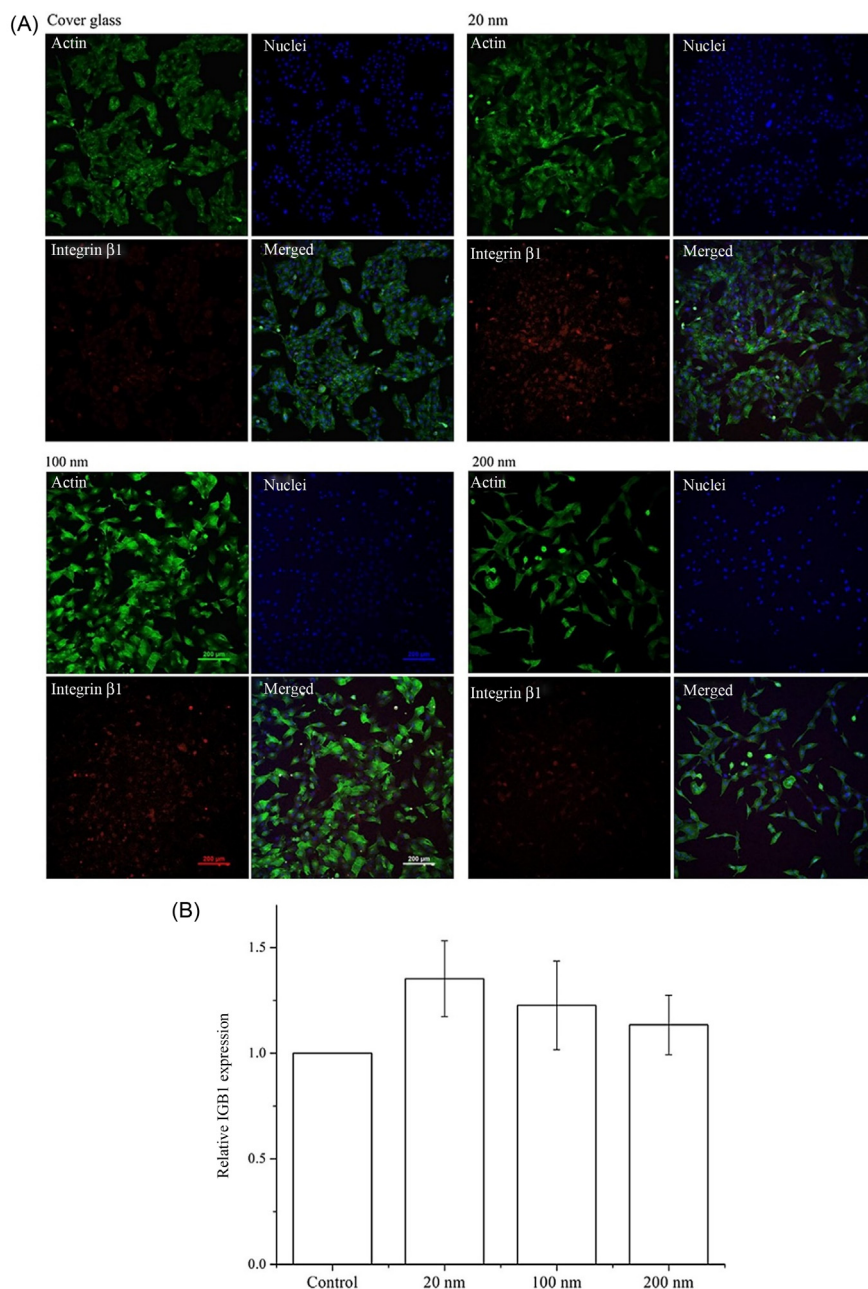


FIGURE 15.1 (A) Immunofluorescence images of MG63 cells after 2 days of adhering to different substrates. In this study, cells were triple stained with cell nuclei (blue), integrin $\beta 1$ (red), and actin filaments (green). (B) Real-time PCR was used to evaluate the relative expression of integrin $\beta 1$. While increasing the pore size, expression of integrin $\beta 1$ in MG63 was reduced and the highest expression was detected in cells cultured on 20 nm sized alumina. The results revealed that alumina is suitable for cell proliferation and adhesion has a smaller pore size. PCR, polymerase chain reaction. Source: Reprinted from Song Y, Ju Y, Morita Y, Song G. Effect of the nanostructure of porous alumina on growth behavior of MG63 osteoblast-like cells. *J Biosci Bioeng* 2013;116(4):509–15 with permission from Elsevier.

precoated with fibrinogen which caused a stronger release of the elastase, myeloperoxidase, and granule enzymes than collagen or serum-coated alumina membranes. On the other hand, osteoblasts' responses including adhesion, viability, and proliferation were mainly influenced by the surface-mediated phagocyte activation and then release of granule components [68].

The initially adsorbed proteins can control the following phenomena, such as activation of blood cells, adherence and activation of the cascade systems of blood [75–77]. Platelets are one of the primary cells that appear at the blood–material interface and also adhesion and activation can be started by communication of platelets with adsorbed proteins [78–80]. Platelet activation contains the release of intracellular granules of platelet, severe variation in platelet appearance, and P-selectin expression on the platelet membrane, promote attraction to fibrinogen, reorganization of the phospholipids of platelet membrane and the formation and release of platelet microparticles [80–82]. It has been known that the activated platelets can control the performance of other inflammatory cells such as monocytes and neutrophils. In another research, Ferraz et al. [83] worked on the impact of alumina membranes with 20 and 200 nm in size porosities and the time sequence of blood activation. In this work, the alumina membranes were incubated with blood from 2 minutes to 4 hours. Overall, the authors have shown that the surface topography of the alumina most reasonably can change protein transition rate, which influences material platelet activation kinetics [83].

Many investigations have exhibited that functionalizing the alumina surface by some specific functional groups could help enhancing cellular and molecular responses. In a study, low molecular weight dicarboxylic acid was added to the alumina surface to modify the chemical properties of it. This study has shown that the carboxyl groups could make a complex with Ca^{2+} on the surface of alumina that leads to forming sites for precipitation of calcium phosphates. These results were approved by cell culture of preosteoblasts (MC3T3-E1 cell line). Overall, this work noted that the addition of special functional groups can enhance cell interaction with the surface of alumina [84]. Besides, a research bone morphogenic protein 2 (BMP2) on porous alumina substrates with various pore sizes, was immobilized to examine the influence of surface topography and chemical agents on the growth behavior of MSCs. In this study, the BMP2-immobilized alumina substrates were characterized and growth behavior and osteogenic differentiation of MSCs were examined. The results showed that functionalizing alumina substrate with BMP2 could improve the adhesion and growth of MSCs. Also, ALP activities and mineralization in cells cultured on BMP2-immobilized alumina substrates were observed significantly higher than the untreated alumina substrates. Finally, the results proposed that surface functionalization of nanoporous alumina substrates with BMP2, could be useful for osteogenic differentiation and cell growth [85].

Some studies have shown that as a result of modifying the chemistry of alumina surface, cells and proteins responses to it will highly improve. Functionalization of alumina surface with some biomolecules enhances different biological properties, including protein adsorption, migration, adhesion, bioactivity, and proliferation of cells [86]. One study has

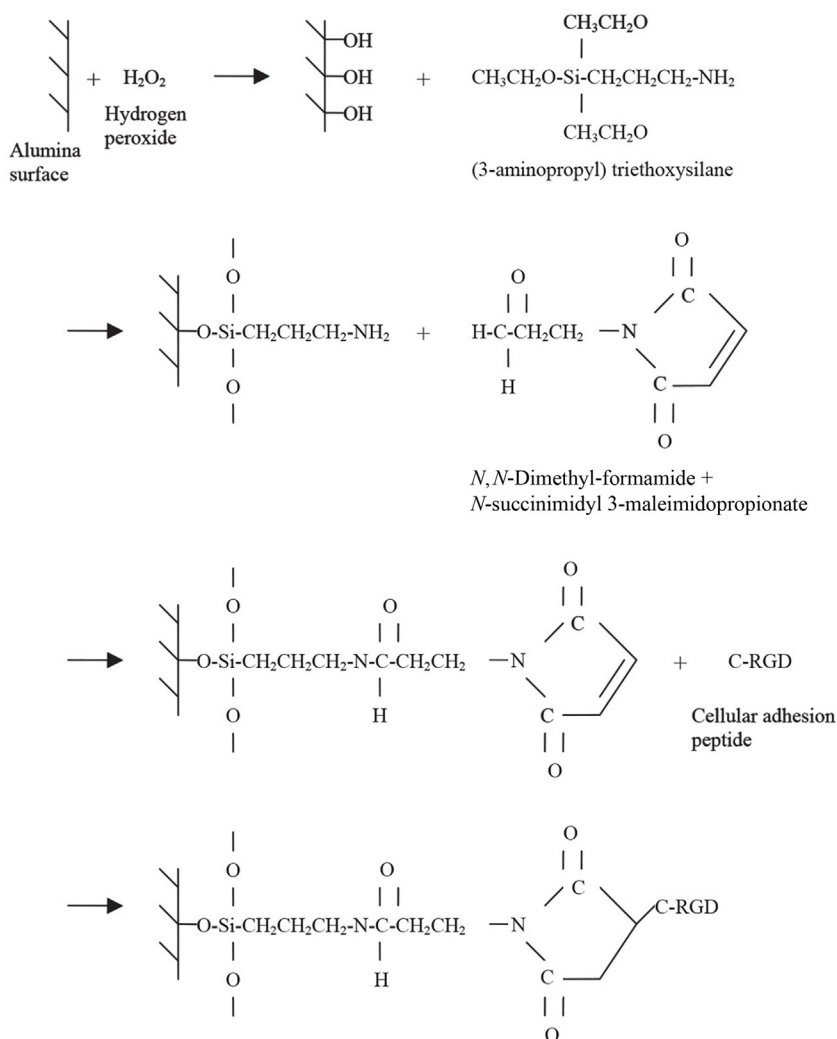


FIGURE 15.2 Reaction scheme for immobilizing RGDC on alumina membrane surface. Using an aminosilane linker, the RGDC peptide immobilized nanoporous alumina membranes were prepared. Silanization was done by incubating alumina membranes in APTES for 2 h. After that, the membranes were washed first with chloroform and acetone and then with deionized water. After washing, the membranes were incubated with the solution of DMF containing *N*-succinimidyl-3-maleimidopropionate, whilst at room temperature. After that the maleimide-grafted membranes were washed using DMF and deionized water. In the next step, membranes were incubated for 2 h at room temperature with the solution of RGDC for a final concentration of 10 mM. APTES, (3-Aminopropyl)-triethoxysilane; DMF, *N,N*-dimethyl-formamide; RGDC, arginine–glycine–aspartic acid–cysteine. Source: Reprinted from Leary Swan EE, Popat KC, Desai, TA. Peptide-immobilized nanoporous alumina membranes for enhanced osteoblast adhesion. *Biomaterials* 2005;26(14):1969–76 with permission from Elsevier.

described that in order to enhance the cellular and molecular responses to alumina, functionalization of its surface by vitronectin and a cellular adhesive peptide could be useful. Also, in another study, to enhance growth osteoblasts on alumina surfaces, the nanoporous alumina surfaces have been altered by adsorbed vitronectin and immobilized arginine–glycine–aspartic acid–cysteine (RGDC) peptide (Fig. 15.2 shows the reaction scheme for the RGDC immobilization on the alumina surface). The data showed that after 1 day of culture in RGDC modified substrates, osteoblasts adhesion was increased and also after 2 days, matrix was produced, while vitronectin modified surfaces could not result in an outstanding improvement in cell adhesion in comparison with the nonmodified surface [87].

Moreover, Kawashita et al. [88] investigated the effects of fibronectin (Fn)-coated disks of hydroxyapatite or alpha-type alumina ($\alpha\text{-Al}_2\text{O}_3$) on the cellular responses of osteoblast-like MC3T3-E1 cells. The outcomes have shown that surface-functionalized with Fn might influence the responses given to biomaterials by inflammatory cells or, in conjunction with other serum proteins, which provoke preosteoblast cell proliferation and differentiation [88]. Fernández et al. [89] used polyvinylpyrrolidone (PVP), to functionalize α -alumina nanoparticles. The results obtained presented that the PVP chains were completely grafted to the alumina surface which caused aiding in enhancing nanoparticles' biocompatibility and measuring their size [89]. Furthermore, Aminian et al. [90] produced a self-mineralization of bone-like material on alumina surfaces employing biomimetic method by carbodiimide-mediated chemoligation technique (see Fig. 15.3).

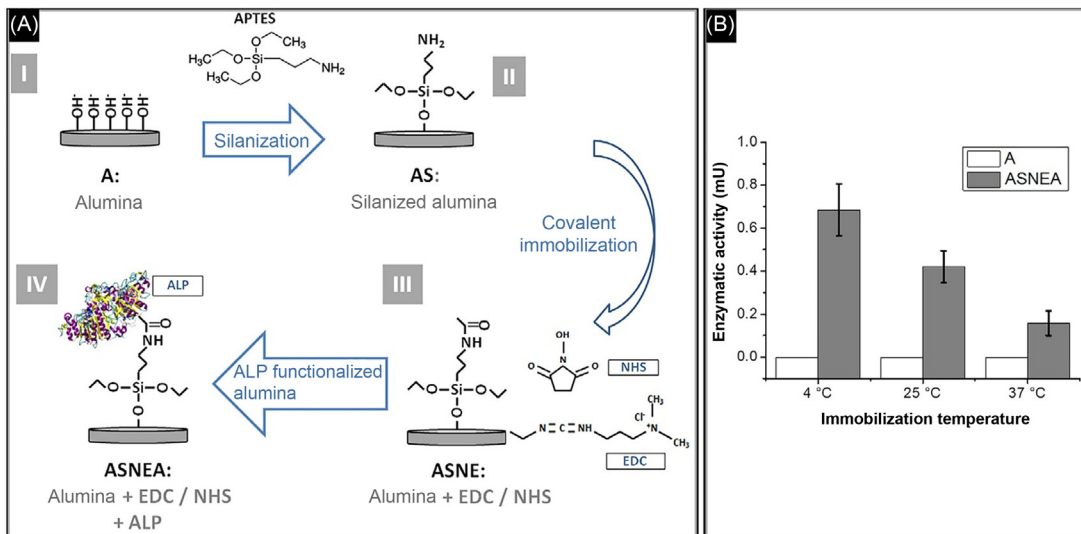


FIGURE 15.3 (A) Functionalization pattern: silanization and covalent immobilization of ALP on alumina substrates with NHS and EDC. (B) Enzymatic activity of alumina discs with immobilized ALP (ASNEA) measured by pNPP assay after immobilization at three temperature points. To control samples, unfunctionalized alumina discs (A) were used. ALP, Alkaline phosphatase; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; NHS, N-hydroxysuccinimide; pNPP, p-nitrophenylphosphate. Source: Reprinted from Aminian A, Pardun K, Volkmann E, Destri GL, Marletta G, Treccani L, et al. Enzyme-assisted calcium phosphate biomineralization on an inert alumina surface, *Acta Biomater* 2015;13:335–43 with permission from Elsevier.

In this study, the mineralizing enzyme ALP was covalently immobilized on the alumina surfaces. The enzymatic activity of immobilized ALP and its mineralization ability were examined by human bone cell culture. In vitro cell analyses showed that ALP-functionalized alumina increased bone cell mineralization. This research has confirmed the ALP-functionalized alumina, because of allowing fast and firm implant osseointegration, have a high ability for the development of bioactive surfaces for purposes like dental and orthopedic materials (see Fig. 15.4) [90].

In one study, alumina nanoparticles were functionalized with (3-methacryloxypropyl) trimethoxysilane (MPS). A vinyl-ester resin monomer was used to polymerize the linked MPS, through a free radical polymerization. This study has shown that the functionalization of

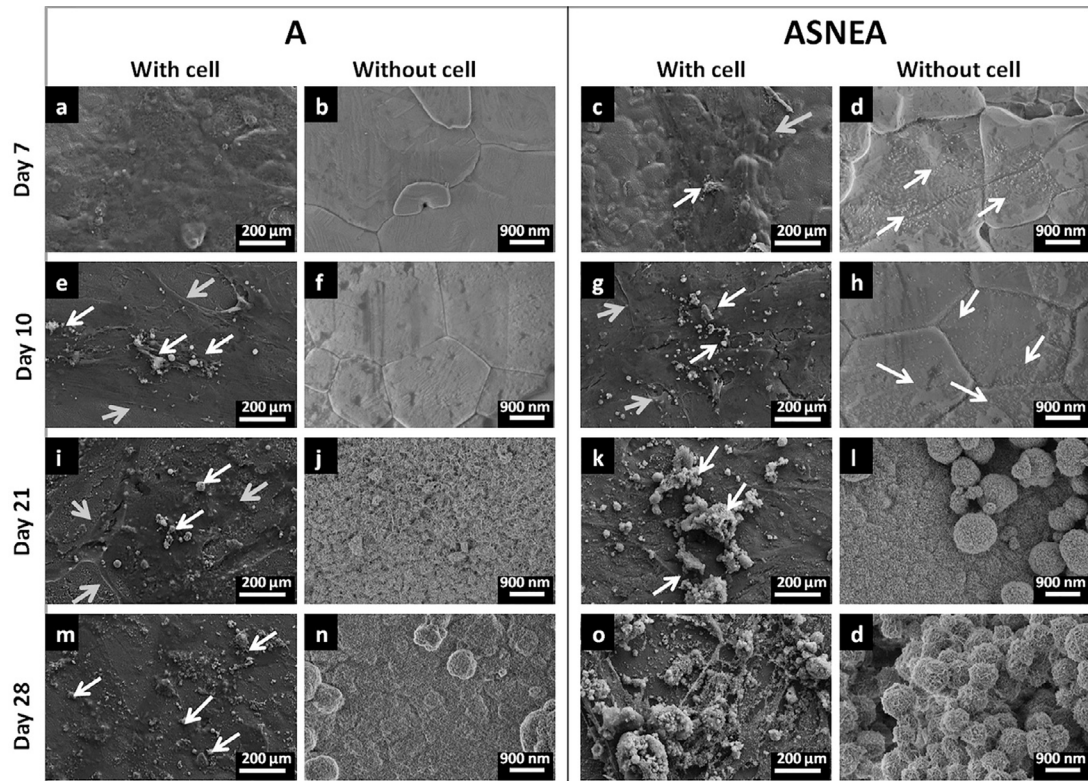


FIGURE 15.4 SEM images of mineral formation with human osteoblast cells after 7, 10, 21, and 28 days of culture in osteoinductive medium: A, nonfunctionalized alumina disks; ASNEA, ALP-immobilized alumina disks; (a, c, e, g, i, k, m, and o) with cells; (b, d, f, h, j, l, n, and p) after cell detachment. Orange arrows indicate the cell area and white arrows indicate mineral globules. On ALP-functionalized samples, small mineralized nodules grown over the surface were identified at days 7 and 10, whereas after days 21 and 28 a thick, uniform mineral layer and large round spherical mineral agglomerates were apparent. On nonfunctionalized alumina samples up to day 10 no mineral deposition was detected, but after 21 days of culturing, mineralized globules were found. Source: Reprinted from Aminian A, Pardun K, Volkmann E, Destri GL, Marletta G, Treccani L, et al. Enzyme-assisted calcium phosphate biomineralization on an inert alumina surface, *Acta Biomater* 2015;13:335–43 with permission from Elsevier.

nanoparticles will lead to forming particle/matrix interfacial bonding that will provide larger, local plastic deformation in the matrix. Also, the results have shown that the modulus and strength could be significantly improved by the addition of the functionalized nanoparticles which has no harmful impacts on the thermal stability of the composite, and the alumina nanoparticles are shielded from dissolution by vinyl ester resin after curing [91]. Besides, Meder et al. [92] investigated the influences of functional groups such as NH_2 , COOH , SO_3H , and PO_3H_2 on the surface of alumina particles on the adsorption of lysozyme, trypsin, and bovine serum albumin. The authors explained that variations in the hydrophilic/hydrophobic properties of the functional groups does not change the amount of adsorbed protein. The results showed that functional groups could manage the communication of protein particles, though it owed to the protein features themselves too [92].

Some study has demonstrated that metal oxide nanoparticles, including aluminum oxide nanoparticles, are widely recognized to have strong antimicrobial properties. Sadiq et al. [93] in their research evaluated the antimicrobial properties of alumina nanoparticles within a wide concentration range (10–1000 $\mu\text{g}/\text{mL}$). This study has shown that only at very high concentrations, alumina nanoparticles have a mild growth-inhibitory effect, that might be due to surface charge interactions between the cells and nanoparticles. Cell wall disruption and severe antimicrobial activities could be restricted by free-radical scavenging behaviors of the nanoparticles. Overall, this study confirmed that alumina nanoparticles may only show mild toxicity toward microorganisms in the environment. In another study, aluminum oxide nanoparticles exhibited growth-inhibiting impacts of 70% on *Pseudomonas fluorescens*, 36% on *Escherichia coli*, and 57% on *Bacillus subtilis* which was related to the direct attachment of the alumina nanoparticles to cell walls of these bacteria [93].

In recent years, many types of research have focused on the physical, chemical, biophysical, and biochemical properties of a cell's surface. It has been shown that most of the surfaces of cells are charged. This charge is owing to the biochemical structure of the cell membranes. So, the examination of a cell surface charge is a vital criterion to understanding the information about the cell's surface [94]. Measurement surface zeta-potential of the cells is necessary to explain the interaction mechanisms of the cell's surface with drugs, antibiotics, and nanoparticles [95,96]. Recently, one research evaluated the zeta-potential change of Neuro-2a tumor cells in the presence of alumina nanoparticles. It has been seen that when the Neuro-2a tumor cells are exposed to metal oxide nanoparticles and inducing apoptosis, zeta-potential changed to negative values [95]. Besides, anticancer properties of aluminum oxide nanoparticles have been reported. In another study by Rajan et al. (2015) [97] aluminum oxide nanoparticles modified by poly-glutamic acid were fabricated and employed as cytotoxic factors to induce cell apoptosis in human prostate cancer cells. This study showed cell cytotoxicity to PC-3 prostate cancer cells by the induction of ROS and consequent mitochondrial dysfunction [97].

Autophagy induction is considered as one of the principal objectives of next-generation vaccines and immunotherapy. For this purpose, the autophagy inducer potential of aluminum oxide nanoparticles has been investigated. In one study, aluminum oxide nanoparticles and cysteine peptidase A and B were conjugated and employed to *Leishmania* vaccine as inducing autophagy in macrophages. The conjugated aluminum oxide nanoparticles were presented that following administration of these nanoparticles rapidly internalized

the *Leishmania-infected* macrophages [98]. Moreover, the aluminum oxide nanoparticles have been used for designing an anti-HIV vaccine to elicit systematic and mucosal immunity. Consequently, aluminum oxide nanoparticles and a peptomer derived from the C4 domain of HIV-gp120 protein were covalently conjugated. The results have shown that prepared nanoconjugate with 300 nm in size was able to elicit an immunologic response in the mucus [99].

Some research has employed alumina nanoparticles as drug delivery systems. For example, in one study ibuprofen loaded with alumina nanoparticles was fabricated by the sol–gel method to increase the bioavailability of this drug. Also, alumina nanoparticles were fabricated by hydrolysis of aluminum oxide alkoxide, then water-insoluble ibuprofen was loaded into the prepared alumina nanoparticles. After, the ibuprofen loaded alumina nanoparticles were characterized. The results showed that the solubility and the controlled release of loaded drugs from aluminum oxide nanoparticles containing ibuprofen was improved. High loading and controlled release efficiency of ibuprofen, are led by high surface area, highly porous structure, and high density of hydroxyl groups on the surface of alumina nanoparticles. Finally, this study showed that alumina nanoparticles can be an efficient drug delivery carrier [100].

15.4 Futures and conclusion

Many studies have concentrated on developing mechanical properties and biocompatibility behavior of alumina; however, some challenges have remained in clinical products. It has been reported that by optimizing some parameters such as the sintering temperature, time and rate, powder processing method, and grain boundary chemistry, properties of alumina will be controlled throughout its fabrication process. Nevertheless, due to outstanding biomechanical and biocompatibility properties of alumina, it has obtained the attention of biomedical specialists in the last few decades, but its practical and clinical aspects are yet to be determined. In addition, despite the presence of many types of research about cellular responses to alumina, full understanding of its biological mechanisms is required.

Some fresh approaches for optimizing alumina surface and achieving better immunological system responses must be provided. Despite the fact that several types of research have been done to improve its long-term clinical use, still, more research with this respect is essential. Many studies have recommended that the biological functions of alumina-based biomaterials can be enhanced by surface nanotopography strategies, but it seems more researches are essential for understanding the influences of applying this strategy on cell behaviors over a prolonged period and the influences of pore sizes of alumina, in a wide range, on the cellular and molecular responses [100]. Also, in future investigations, other biological factors that can have influence on managing immunological responses to alumina surfaces should be taken into account.

Due to a few investigations that have concentrated on the influences of additional functional groups on alumina surfaces' interactions with proteins' enhancement, there is a critical requirement to investigate the influences of other chemical surface modification approaches on its biological functions. Furthermore, among the literature, there is no influential database

about specific mechanisms associated with foreign body reactions to alumina substrates, which should be considered by immunologists and biologists. It is a strong fact that using more biomaterials for tissue regeneration, requires more knowledge of the principles of cell response to biomaterials.

Alumina nanoparticles have been considered as suitable nanomaterials that can be used in biomedical science and biotechnology applications, due to requirements of developing biomedical science swiftly, for utilizing new types of material. Nevertheless, some examined toxicity may cause limiting the progress of alumina nanoparticles in some applications such as proteins, drugs, or carriers for therapeutic nucleic acids' intracellular delivery. Accordingly, it has been reported that the unavoidable approach to generate biocompatible alumina nanoparticles is using the surface modifications approach. So, it seems that knowing other fields of biomedical sciences development is important for future researches of alumina nanoparticles. It means these nanoparticles can be employed in other areas with fewer investigations including soft tissue engineering, in vivo imaging, nucleic acid delivery, and targeted therapy.

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Biocompatibility of graphene quantum dots and related materials

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Abbreviations

Amine-modified graphene	G-NH ₂
Carbon nanotube	CNT
Dextran	DEX
Graphene oxide	GO
Graphene quantum dot	GQD
Nanodiamond	ND
Reduced graphene oxide	rGO

16.1 Introduction

Graphene is a two-dimensional crystalline allotrope of carbon, with a single layer of sp² hybridized carbon atoms and is arranged in a hexagonal lattice. They are materials with single atom thickness, but they are the hardest material. This property can be utilized to convert them into thin sheets. Recently, they have held great importance due to their properties like large surface area, good strength, excellent thermal conductivity, electrical conductivity, excellent transmittance of light, lower toxicity, and low cost [1]. The graphene family of nanomaterials can be classified into graphene (single layer, bilayer, and multilayer), graphene oxide (GO), reduced graphene, reduced GO (rGO), graphene quantum dots (GQDs), graphene nanoribbon, graphene nanosheets, and chemically-modified graphene based on a number of layers, amount of oxygen present, purity, surface chemistry, or functional groups and their composition [2]. GQDs are a new class of graphene-based quantum dots discovered in 2008 by Ponomarenko and Geim and show bright fluorescence under ultraviolet (UV) light and the fluorescence depends on their size, shape, and functional groups. They are extremely small, usually less than 20 nm size, and can cross

blood–brain barrier. Now they are explored as good candidates for drug delivery, diagnosis, bioimaging, and sensing applications [3–5].

The materials for any biological application must be nontoxic and compatible with the body. The toxicity leads to serious health issues, and even causes death. Thus the biocompatibility of GQDs and related materials should be considered before using for biological applications. Different graphene-based materials possess different toxicity levels and there are some factors that determine the toxicity. GQDs and related materials are used as therapeutic agents, drug carriers, biosensing and bioimaging agents, diagnostic tools, etc. For every material, for any above application, in vitro and in vivo toxicity studies are performed to ensure safety and biocompatibility [6].

16.2 In vitro biocompatibility studies

In vitro biocompatibility study means, toxicity of structures that are tested on different cell lines. In vitro cell line studies are comparatively simple than in vivo animal studies. In vitro cell line studies of GQDs and other graphene-related compounds are discussed in the following section.

16.2.1 In vitro biocompatibility study of graphene quantum dot

GQD can be synthesized by the decomposition of GO in dimethyl formamide by solvothermal method, and the toxicity of these GQDs can be tested on prostate cancer cell lines, DU-145 and PC-3. The maximum tolerable toxicity for DU-145 cells was found to be 100 $\mu\text{g/mL}$. Below this level, there is no sign of cell viability and above this level, the cell viability decreases. But, at a concentration of 400 $\mu\text{g/mL}$, the viability still remains at 80% and similar results are observed in PC-3 also. Thus GQDs are safe in both cell types [7]. The interaction of GQDs with blood cells such as monocytes and granulocytes can be studied. GQDs are taken into the cells by caveolae endocytosis and accumulate in endoplasmic reticulum and nucleus. Finally the study concluded that, GQDs do not interfere the cell proliferation [8].

GQD can be synthesized from salicylic acid by free radical polymerization under UV irradiation, and that can be used for in vivo and in vitro bioimaging. OCM-1 cell line can be used to evaluate its in vitro cytotoxicity. GQD concentration from 0 to 500 $\mu\text{g/mL}$ was treated with OCM-1 cell lines and the metabolic activity was examined. After 24 hours incubation, cell viability was not affected even at a higher concentration (500 $\mu\text{g/mL}$) [9]. GQDs can be prepared by the exfoliation of carbon fiber and it can be carboxylated by treating with citric acid. And the toxicity of this carboxylated GQDs can be evaluated on different cell lines including KB, MDA-MB231, A549 cancer cells, and Madin–Darby canine kidney (MDCK) normal cell line. Carboxylated GQDs are easily taken by all the cells and no toxicity or morphological differences were observed [10].

Blue fluorescence emitting GQDs can be synthesized from L-glutamic by its pyrolysis at 220°C. They are easily uptaken by KB cells without any toxicity, and are also nontoxic in MDCK cells. Their hemolytic activity is tested by keeping different concentrations of

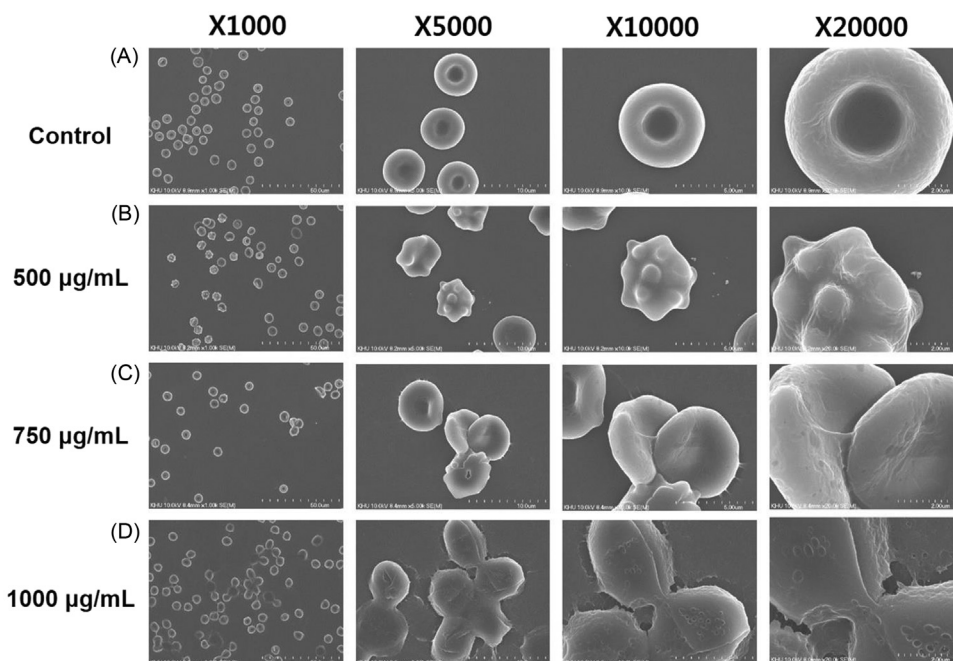


FIGURE 16.1 SEM images of RBCs exposed to different concentrations of GQDs in four different magnification powers ($\times 1000$, $\times 5000$, $\times 10000$, and $\times 20000$). (A) Control, (B) RBCs in 500 $\mu\text{g}/\text{mL}$ of GQD, (C) RBCs in 750 $\mu\text{g}/\text{mL}$ of GQD, (D) RBCs in 1000 $\mu\text{g}/\text{mL}$ of GQD. GQDs, Graphene quantum dots; RBCs, red blood cells. Source: Reproduced with permission from Nafiujjaman M, Kim J, Park H-K, Lee Y-K. Preparation of blue-color-emitting graphene quantum dots and their in vitro and in vivo toxicity evaluation. *J Ind Eng Chem* 2018;57:171–80. doi:<https://doi.org/10.1016/j.jiec.2017.08.019>. ©2018, Elsevier.

GQDs in red blood cells (RBCs) for 2 hours. Fig. 16.1 shows scanning electron microscopy (SEM) images of RBCs exposed to different concentrations of GQDs; the RBC remains as such in control, and GQD exposed samples show cell membrane damage and cell–cell fusion [11].

The membrane permeability of different sized GQDs can be evaluated; 3 and 12 nm GQDs are used for the same. Small GQDs penetrated into MDCK cells by lipid raft-mediated transcytosis [12]. Similarly, the penetration of GQD into human stem cells can be studied without affecting cell viability and proliferation [13]. In vitro cytotoxicity of different concentrations of (0–500 $\mu\text{g}/\text{mL}$) selenium-doped GQDs can be tested on HeLa cells. After 24 hours incubation, the cell viability was evaluated, and there was no significant reduction in cell viability [14].

16.2.2 In vitro biocompatibility study of graphene derivatives

The interaction of graphene nanoparticles with RBCs is a very important one; the hemolytic activity is to be evaluated to ensure safety. GO and graphene sheets exhibit hemolytic

activity in RBCs but, GO is more potent than graphene sheets. This is due to the electrostatic interaction between positively-charged phosphatidylcholine on RBC surface and negatively-charged groups on GO surface. Since graphene sheets have lower oxygen groups, it does not induce hemolysis, but leads to hemagglutinin. To reduce the hemolytic effect of GO, some techniques have been developed; biocompatible chitosan-coated GO can reduce the electrostatic interaction and thus, hemolysis can be reduced [15].

Different concentrations of GO can be prepared and its biocompatibility is evaluated on human fibroblast cells, and it induces dose-dependent apoptosis [16]. GO can be synthesized by modified Hummer method, and the prepared GO suspension can be centrifuged to collect both supernatant and sediment. In vitro toxicity study of the above prepared three samples of GO can be performed with A549 cell lines. The study states that GO can increase reactive oxygen species (ROS) levels even at low concentrations without affecting cell viability. Thus it can be concluded that, GO can induce oxidative stress and lead to cell toxicity. Fig. 16.2 shows optical microscopic images of A549 cells treated with three different GO samples and untreated control cells. There are no detectable changes observed in treated cells and control [17]. Biocompatibility and cellular toxicity of GO in intraocular level was performed and the study reports that, there is no significant effect on human retinal pigment epithelial cell morphology, viability, membrane integrity, and apoptosis, and thus GO has good intraocular biocompatibility [18].

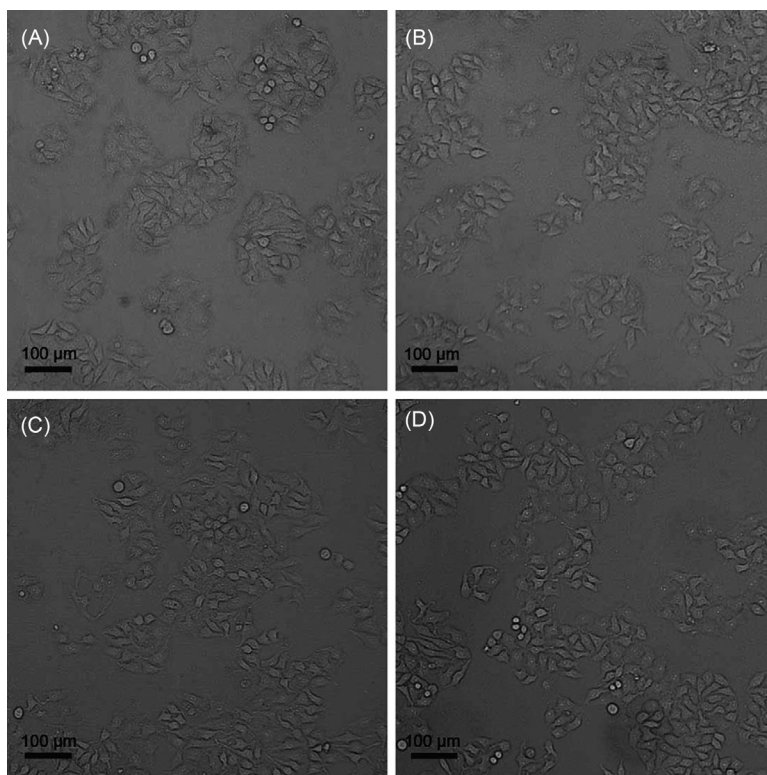


FIGURE 16.2 Optical microscopic images of A549 cells treated with different samples of GO (A, B, C) and control (D). GO, Graphene oxide. Source: Reproduced with permission from Chang Y, Yang S-T, Liu J-H, Dong E, Wang Y, Cao A, et al. In vitro toxicity evaluation of graphene oxide on A549 cells. *Toxicol Lett* 2011;200:201–10. doi:<https://doi.org/10.1016/j.toxlet.2010.11.016>. ©2011, Elsevier.

GO and carboxylated graphene nanoplatelets can be treated with HepG2 cell lines, the particles penetrate the phospholipid bilayer of the cell membrane and then enter into the cell, exhibiting dose and time-dependent ROS production [19]. The use of reducing agents such as hydrazine hydrate and hydroquinone for the synthesis of rGO leads to toxicity [6]. Toxicity of GO and rGO can be compared by culturing with human glioma cell lines U87 and U118 and the result states that, rGO is more toxic than GO [20]. The toxicity of N-doped GQD (N-GQD) and GO on RBCs can be compared. The hemolytic activity, morphological changes, and ATP content of RBCs after the exposure can be evaluated. GO reduces membrane integrity due to lipid bilayer extraction and leads to hemolysis, but N-GQDs show only structural changes on RBC membrane and are found as aberrant-shaped RBCs on microscopic examination [21].

16.3 In vivo biocompatibility studies

In vivo studies are real animal studies, which gives more accurate results than cell line studies. Here, the biocompatibility studies are tested on mice, zebrafish, some nematodes, etc.

16.3.1 In vivo biocompatibility study of graphene quantum dots

The toxicity of GQDs in rat lungs after intravenous administration can be evaluated by histological examination of the lung tissue. The tissues can be examined for any damages, pathological changes, inflammation, or necrosis due to GQD, and no gross changes or abnormalities were observed in lower concentrations. At higher GQD concentrations, alveolar septa were thickened [22]. Zebrafish *Danio rerio* can be used for in vivo toxicity studies of graphene. Polylactic acid and fluorescein *o*-methacrylate functionalized graphene can be microinjected into zebrafish embryo and exhibited good biodistribution, and there were no detectable toxicity or abnormalities observed [23]. The effect of prolonged exposure of N-GQDs on a nematode starin *Caenorhabditis elegans* and its progeny can be evaluated. The locomotion behavior and number of offspring are taken as evaluation parameters, and there is no effect for the nematode locomotion after prolonged exposure and also nontoxic to offspring [24]. Long-term intravenous administration of carboxylated GQDs into mice leads to accumulation in the liver, lungs, kidneys, spleen, and biochemical evaluation of treated mice serum shows no significant toxicity [10].

The effect of GQD toxicity in male mouse reproduction and offspring health can be investigated by oral gavage or intravenous administration. The male mouse was housed with the female mouse and its reproductive health was studied by evaluating testosterone levels, sperm motility, α -glucosidase activity in seminal plasma, etc. After the gestational period, the mouse gave birth to healthy pups in three subsequent litters. Thus GQD shows less toxicity to reproductive cells and no effects in the reproductive system. Fig. 16.3 gives a schematic representation of the experiments and evaluation on the mouse [25].

16.3.2 In vivo biocompatibility study of graphene derivatives

Administration of GO into mice leads to pulmonary toxicity, pulmonary edema fibrosis, granulomatous lesions, and inflammatory cell infiltration, and all these effects depend on

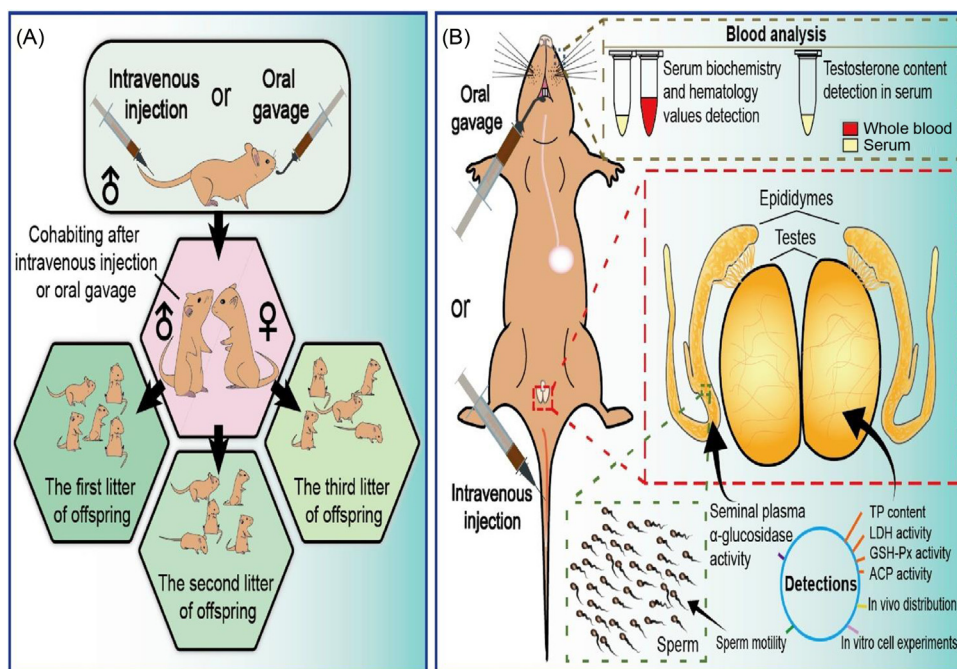


FIGURE 16.3 Schematic representation of evaluation GQD toxicity in male mouse reproduction and offspring health. (A) Administration of GQD and evaluation of mouse sexual behaviors and offspring health. (B) Toxicity studies of GQD in male mouse germ cells. GQD, Graphene quantum dot. Source: Reproduced with permission from Zhang D, Zhang Z, Wu Y, Fu K, Chen Y, Li W, et al. Systematic evaluation of graphene quantum dot toxicity to male mouse sexual behaviors, reproductive and offspring health. *Biomaterials* 2019;194:215–32. doi:https://doi.org/10.1016/j.biomaterials.2018.12.001. ©2019, Elsevier.

the concentration of GO [16,26]. Different concentrations of GO can be administered into mice to evaluate its *in vivo* toxicity. The results conclude that, GO induces lung granuloma in mice and it is found that toxicity is dose dependent [16].

GO induces certain cardiovascular problems, the study was performed in zebrafish embryo. At a concentration of 0.4–1 mg/mL, GO causes cardiotoxicity, increased heart-beat, and some other cardiovascular defects that are shown in Fig. 16.4 [27].

Intravenous administration of GO into mice causes pulmonary thromboembolism due to thrombogenic nature of GO and also shows aggregation of human platelets [28]. To study the intraocular biocompatibility and cytotoxicity of GO, it is injected into the rabbit eye as an intravitreal injection. The injection did not cause any changes on eyeball appearance [18]. The effect of graphite nanoplatelets on nematode *C. elegans* was studied by taking longevity and reproductive capacity as toxicity parameters. Different concentrations of graphite nanoplatelets did not cause nematode death and then concluded that, there is no acute toxicity [29]. Toxicity of rGO can be evaluated in mice after single intravenous administration; rGO did not show toxicity in mice hippocampus [30]. Polyethylene glycol (PEG)-functionalized rGO shows toxic effects in brain astrocytes and endothelial cells in rats. PEG causes downregulation of some brain components and the

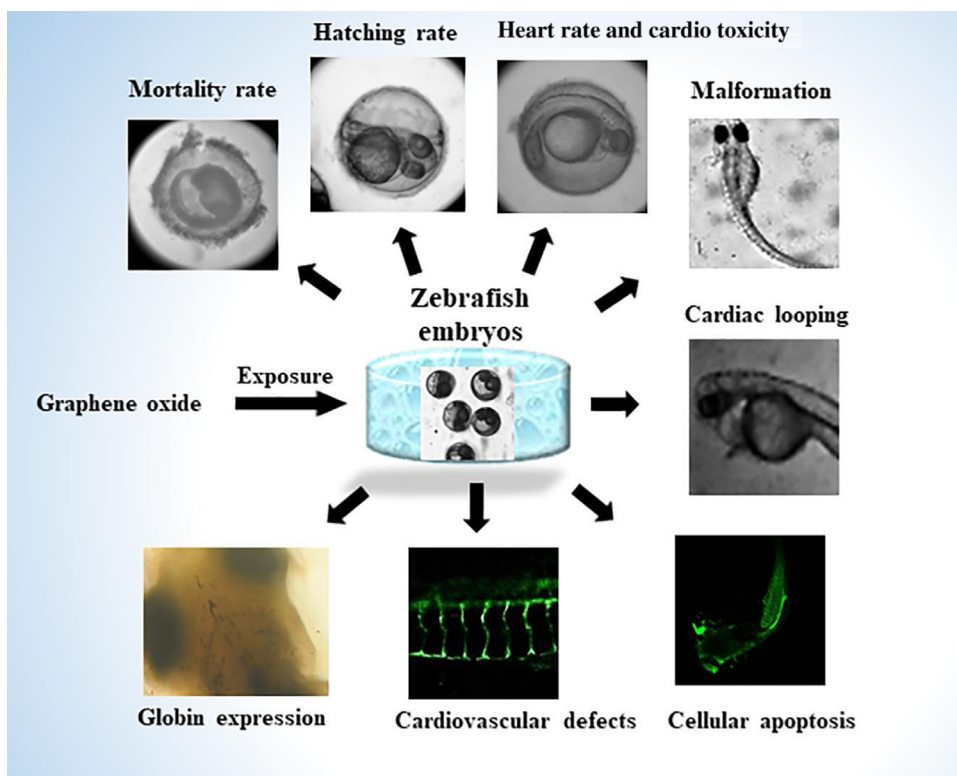


FIGURE 16.4 Shows exposure of GO into zebrafish embryo and resulting abnormalities in the cardiovascular system. GO, Graphene oxide. Source: Reproduced with permission from Bangeppagari M, Park SH, Kundapur RR, Lee SJ. Graphene oxide induces cardiovascular defects in developing zebrafish (*Danio rerio*) embryo model: in-vivo toxicity assessment. *Sci Total Environ* 2019;673:810–20. doi:<https://doi.org/10.1016/j.scitotenv.2019.04.082>. ©2019, Elsevier.

production of ROS, which induces oxidative stress [31]. An in vivo comparative toxicity study of GO and rGO was performed and the results concluded that, both rGO and GO can reduce the tumor volume and weight [20].

The effects of GO toxicity can also be tested on fresh water zooplankton *Ceriodaphnia dubia*. Both acute and chronic exposure of GO affects their survival and inhibits feeding and reproduction, and GO accumulates in the gut tract as shown in Fig. 16.5 [32].

16.4 Biocompatibility study of other carbon nanostructures

16.4.1 Biocompatibility study of carbon nanotube

Physicochemical characteristics of carbon nanotube (CNT) have an important role in their toxicity. Length, diameter, surface area, agglomeration, presence of catalyst residues, and chemical functionalization can determine the CNT toxicity. Functionalized CNTs are less toxic and more biocompatible than nonfunctionalized ones. Intravenous administration of well-

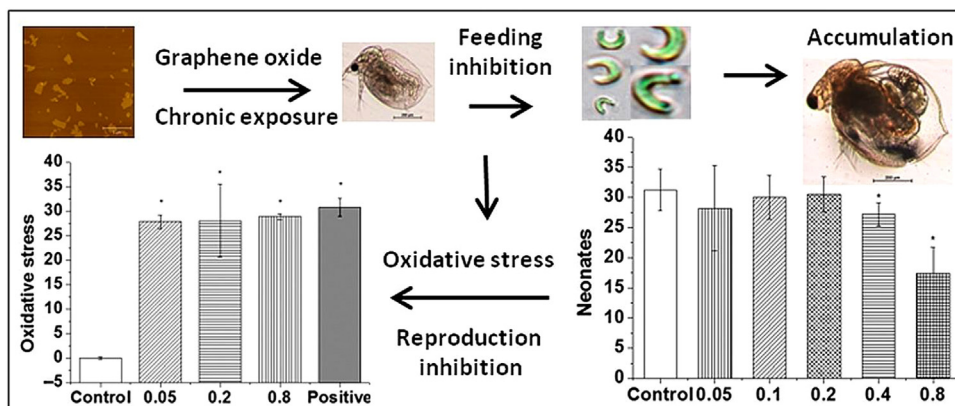


FIGURE 16.5 Acute and chronic exposure of GO on *Ceriodaphnia dubia* and the effects in reproduction and feeding. GO, Graphene oxide. Source: Reproduced with permission from Souza JP, Venturini FP, Santos F, Zucolotto V. Chronic toxicity in *Ceriodaphnia dubia* induced by graphene oxide. *Chemosphere* 2018;190:218–24. <https://doi.org/10.1016/j.chemosphere.2017.10.018>. ©2017, Elsevier.

functionalized CNT with PEG-like biocompatible coating into animals shows reduced in vivo toxicity than unfunctionalized pristine CNT. The toxicity can be evaluated by measuring cell viability, cell inflammation, and production of ROS. The catalysts involved in the synthesis of CNT such as nickel, cobalt, iron, etc. may be sometimes present on CNT surface or within them, and these metal remnants may cause oxidative stress and reduce cell viability. Thus the purification of CNT during synthesis has a major role to reduce toxicity. High-temperature annealing, acidic treatment by reflux, or steam-purification can be adopted during synthesis to remove impurities, thus toxicity due to impurities can be eliminated [33–36].

Surface defects on CNT such as incomplete bonding defects, presence of SP³ hybridized carbon, some functional groups, etc. can induce pulmonary toxicity and genotoxicity. The length of CNT has a critical role, the effect of CNT length on cell inflammation was studied in peritoneal mesothelium. Long fibrous CNT shows significant inflammation and not by short fibrous CNT. Similarly, the diameter of the CNT also can determine the toxicity. The effects of two multi-walled carbon nanotubes (MWNTs) with the same length having different diameters of 9.4 and 70 nm were studied on murine alveolar macrophages. Thin MWNT shows more cytotoxicity than thick MWNT [37,38]. The toxicity of single-walled carbon nanotube (SWNT) can be studied on the chicken embryonic spinal cord. SWNT up to 30 µg/mL concentration can be incubated with neuroglial cultures. The study results concluded that SWNTs show toxic effects on glial cells and neurons at a higher concentration [39]. CNT suspended by surfactants can be orally administered and is safe even at an ultra-high doses up to 1000 mg/kg. Intratracheal administration of nonfunctionalized CNT leads to aggregation in the lungs and it causes lung toxicity and inflammation [36].

The toxicological impact of pristine and functionalized MWNTs was evaluated in neural cells isolated from two different regions of the brain, the striatum and the frontal cortex of a fetal rat brain. The cells did not show an evident detrimental effect on exposure to MWNTs at doses up to 100 µg/mL [40]. Several studies already proved the preferentiality

of functionalized CNTs to be accumulated within the microglial cells when introduced in the brain. Considering the limited knowledge of CNT and microglial cell interactions and the key role of these microglial cells in the central nervous system (CNS), a study was performed. The study focused on understanding, whether the microglial cell and the CNT interactions are triggering any proinflammatory signaling or altering the physiological functions of the microglial cells. The study demonstrated that the accumulation of functionalized CNTs in the microglial cells is not affecting the basic physiological functions of these cells and inducing any inflammatory responses [41].

16.4.2 Biocompatibility study of fullerene

Cytotoxicity assay of different concentrations of polyvinylpyrrolidone (PVP)-fullerene and Pox-fullerene can be performed in CATH. neuronal cells. After 24 hours incubation study, the fullerene-polymer complexes were found to be nontoxic to neuronal cells up to 50 μ M concentration [42]. The particle size of fullerene has an important role in its toxicity determination, particles with <100 nm possess higher toxicity than microparticles. One group of rats is exposed to 55 nm diameter fullerene and another group of rats is exposed with 0.93 μ m particles by nasal inhalation for 3 hours per day for 10 consecutive days. The microparticles of fullerene did not show any inflammation or toxicity in the lungs [43].

Oral administration of fullerene shows any absorption, a major portion is excreted in feces and a small amount of fullerene is observed in urine and it indicates that some fullerene can pass through gut wall [44]. Fullerite is the mixture of C₆₀ and C₇₀ in 0.5% aqueous solution of sodium carboxyl methyl cellulose containing 0.1% Tween 80. The acute toxicity of fullerene can be evaluated after oral administration of this solution in rats for 14 days at 2000 mg/kg doses. No toxicity and no lethality were observed [45]. Polyalkylsulfonated fullerene can be orally administered into mice at 50 mg/kg at a time (acute). The observations and results concluded that the formulation is nontoxic. Similarly, a subacute exposure (50 mg/kg daily for 12 days) of the same formulation was also given to mice and it was also nontoxic [46].

16.4.3 Biocompatibility study of carbon dot

Even though carbon dots are good biocompatible nanostructures, the studies so far on carbon dots toxicity with limited cell lines are unsatisfactory. The photoinduced toxicity studies and the cytotoxicity studies conducted on the unicellular eukaryotic model yeast cells (*Pichia pastoris*) have revealed the dose-dependent toxic nature of carbon dots with yeast cells. Upon light exposure, carbon dots exhibited enhanced growth inhibition and ROS generation dose-dependently [47]. The toxicity study of carbon dots on rare minnow (*Gobiocypris rarus*) embryos at different developmental stages has resulted in imbalanced gene expression and developmental defects, also induced oxidative stress indicating significant developmental toxicity of rare minnow embryos on carbon dot exposure. This study also results in concentration-dependent developmental toxicity in rare minnow embryos on carbon dot exposure [48]. The mice subjected to different treatment concentrations of

carbon dots on single inhalation exposure has resulted in lung and liver injury along with necrosis and inflammation and has exhibited an enhanced rate depending on time and concentration of exposure. A theoretical basis regarding the respiratory toxic effect of carbon dots can be obtained from this study [49].

16.4.4 Biocompatibility study of nanodiamond

In vitro and in vivo biocompatibility and toxicity studies of nanodiamond (ND) were performed to ensure its suitability in biomedical applications. Their biocompatibility is superior over single-walled CNTs and carbon blacks. In vitro toxicity of ND can be tested by MTT assay and ATP production assay and reported that they are nontoxic. Their toxicity was also compared with other carbon nanostructures such as MWNT, SWNT, and carbon black on neuroblastoma cells and macrophages, and the order of toxicity was found as SWNT > MWNT > carbon black > ND [50]. In another study, the toxicity of CNT and ND is compared to human lung A549 epithelial cells and HFL-1 normal fibroblasts. And the results revealed that CNT possesses higher toxicity than NDs. Then the toxicity of carboxylated CNT and NDs were compared. Carboxyl-modified NDs of 5 and 100 nm did not affect cellular viability and protein expression. But, carboxyl-modified CNT exhibits toxicity under similar conditions [51].

Fluorescent NDs can be intravenously administered into a rat; a single high dose infusion was found to be safe in the rat as the hematological and biochemistry data were normal [52]. The cytotoxic effects of fluorescent NDs can be studied on mouse hippocampal neurons and mouse dorsal root ganglion neuron to evaluate the toxicity effects in CNS and peripheral nervous system (PNS) respectively. Different concentrations of fluorescent NDs are then applied to neurons. After treatment, neuron morphology quantification software neurology can be used to determine the number of neurons. The study result concluded that fluorescent NDs do not cause any changes in neurons. Activated caspase-3 is the apoptotic marker in the neurons; after fluorescent ND treatment there is no increase in the caspase-3 in hippocampal neurons. Thus it is concluded that fluorescent NDs do not cause cytotoxicity up to 250 $\mu\text{g/mL}$ [53]. If NDs are synthesized from the detonation process, they are obtained as low-density powder, and there is a chance for environmental pollution during manufacturing by spreading into the air. Thus respiratory toxicity of NDs is studied in mice by intratracheal instillation. The results show that there is no significant adverse reaction with ND during the study period and no lipid peroxidation was observed [54]. ND hydrosol can be orally administered for a long time period to evaluate its toxicity in the next generation too. For this, 0.002–0.05 wt.% ND hydrosols are administered to mice instead of water in the diet for 3–6 months. Thus a total of 16–450 mg of ND is delivered into mice, with no deaths reported and internal organs like liver, heart, lung, kidney, and pancreas are not affected. Moreover, the substitution of water with ND hydrosol did not affect the offspring; the treated mice produced healthy offspring [55]. Subcutaneous administration of ND for a 3 month period did not cause any inflammatory signs in mice [56]. From these studies, it is understood that the administration of ND through different routes did not cause any toxicity and thus they show good biocompatibility [57].

16.5 Approaches to reduce toxicity

The biocompatibility of graphene nanoparticles is necessary for biological applications. But some in vitro and in vivo studies report toxicity, thus the toxicity can be reduced before biological applications. Functionalization with biocompatible polymers, coating with biocompatible materials, and green synthesis are some techniques to reduce the toxicity of graphene nanoparticles which are discussed below.

16.5.1 Green synthesis

The reducing agents used for the preparation of rGO lead to toxicity; hence green synthetic methods have been developed to eliminate toxicity [6]. Since L-ascorbic acid can act as a capping agent, it can prevent aggregation of reduced graphene sheets when it is used as a reducing agent of GO [58]. Similarly, the toxicity of reduced graphene sheets can be eliminated when L-tryptophan is used as the stabilizing agent during synthesis. Use of fructose, glucose, and sucrose for the synthesis of graphene nanosheets can reduce toxicity and form very stable dispersion in water [59]. Extracts of leaves of *Mesua ferrea* and *Colocasia esculenta* and aqueous extract of orange peel can be used as reducing agents for GO reduction [60].

16.5.2 Coating/functionalization

Graphene-derived nanoparticles can be coated with biocompatible polymers; it can reduce the toxicity and enhance the stability, solubility, and retention time in biological fluids. PEGylated graphene nanosheets are easily uptaken by tumor cells and have excellent photothermal properties [61].

Dextran (DEX), a hydrophilic polymer can be used for coating the surface of GO to minimize the toxic effects of GO. This can be studied in HeLa cells by investigating the cell proliferation with different concentrations of GO and GO-DEX. In the presence of GO, the cell density was found to be reduced rather than controlled. When DEX was coated with GO, the cell viability was retained. Therefore the study can be concluded that, DEX-coated GO can minimize the toxic effects of GO.

DEX-coated graphene nanosheets can reduce histamine release and shows antiallergic activity [62]. Chitosan and DEX-modified graphene nanoparticles can reduce the hemolytic activity [63]. Amine-functionalized graphene nanomaterials are safe as they do not cause hemolysis, platelet aggregation, or pulmonary embolism [63]. PEGylated GO are incorporated with injectable hydrogel and administered into mice, and no significant toxicity was observed [64].

The thrombogenic nature of GO can be minimized by converting it into amine-modified graphene (G-NH₂). The resulting G-NH₂ has cytoprotective action and has no effect on human platelet stimulation and it does not induce pulmonary thromboembolism in mice [28].

16.6 Conclusion

In vitro and in vivo toxicity studies of GQDs and other graphene-derived materials are discussed in the review. Some studies report them as nontoxic materials and in some studies they exhibit cytotoxicity and reduce cell viability. Their size, shape, and functionalization have an important role in toxicity determination. Pulmonary toxicity, granulomatous lesions, pulmonary edema fibrosis, and thromboembolism are some reported toxic reactions. Some ideas are developed to reduce the toxic effects; green synthesis of graphene-derived materials can minimize the toxicity to some extent. Similarly, the functionalization or coating with biocompatible polymers can also reduce their toxic effect. Further research studies are required to detect unrevealed toxic effects and newer techniques are needed to minimize those toxic effects, and thus the safety of graphene nanoparticles in biological applications can be ensured.

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Cellular response to calcium phosphate cements

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17.1 Introduction

Calcium orthophosphate (COP) ceramics have been investigated as bone repair materials for many decades. The first *in vivo* application of these materials was accomplished in 1920 to test tricalcium phosphate (TCP) performance as a bone substitute [1]. Since this first attempt, various COPs were investigated on animals to shed light on their effect on the healing behavior of bones. Calcium phosphate (CP) cements (CPCs) are currently receiving a great deal of interest especially for the hard tissue repair, augmentation, and regeneration applications [2,3] due to their attractive characteristics such as biocompatibility, ease of shaping, osteoconductivity, and biodegradability (Fig. 17.1) [4,5].

CP minerals, a major inorganic part (~60%) of the natural human bones, are compounds of phosphate anions and calcium cations. Chemical composition of the CPs is very similar to mineral fraction of bone (Table 17.1). The chemical content of human bone, dentin, and enamel differ from each other in terms of content, chemical composition, and design. These differences are summarized in Fig. 17.2.

CPCs can be evaluated into two main groups as apatite and brushite cements (Fig. 17.3). Hydroxyapatite (HA) and calcium-deficient HA (CDHA) are the best known

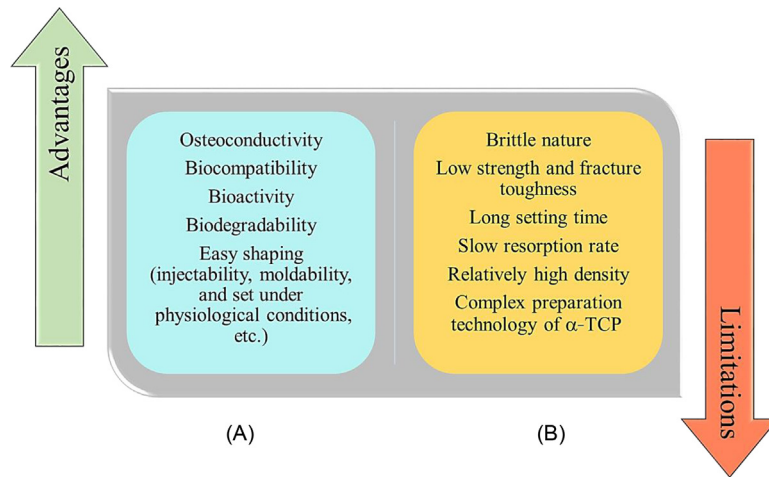


FIGURE 17.1 Fundamental (A) advantages and (B) disadvantages of CPCs. *CPCs*, Calcium phosphate cements.

TABLE 17.1 Chemical content comparison of hydroxyapatite (HA) with inorganic part of human dentin, bone, and enamel tissues [6–9].

Composition (wt.%)	Dentin	Bone	Enamel	HA
Calcium	35.1	34.8	36.5	39.6
Phosphorus (as P)	16.9	15.2	17.7	18.5
Molar ratio of Ca/P	1.61	1.71	1.63	1.67
Carbonate (as CO_3^{2-})	5.6	7.4	3.5	—
Magnesium	1.23	0.72	0.44	—
Sodium	0.6	0.9	0.5	—
Pyrophosphate (as $\text{P}_2\text{O}_7^{4-}$)	0.10	0.07	0.022	—
Fluoride	0.06	0.03	0.01	—
Potassium	0.05	0.03	0.08	—
Chloride	0.01	0.13	0.30	—
Sum of the inorganic content	70	65	97	100
Sum of the organic content	20	25	1.5	—
Water content	10	10	1.5	—
Trace elements: Sr^{2+} , Ba^{2+} , Zn^{2+} , etc.				
Ignition products at 800°C	β -TCP + HA	HA + CaO	β -TCP + HA	HA

TCP, Tricalcium phosphate.

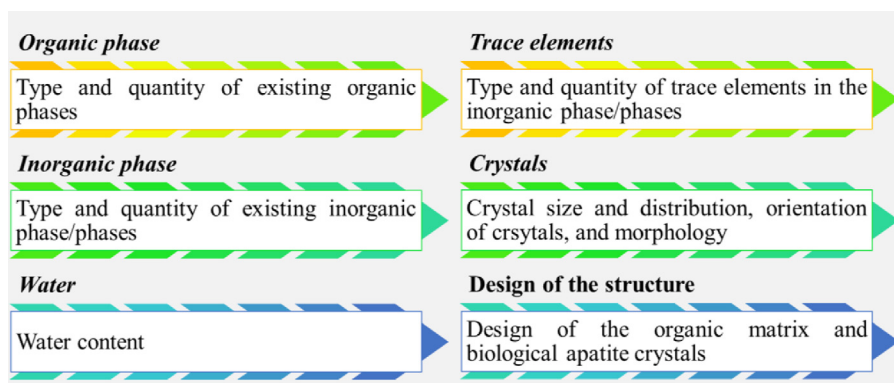


FIGURE 17.2 Essential differences between human bone, dentin, and enamel [10].

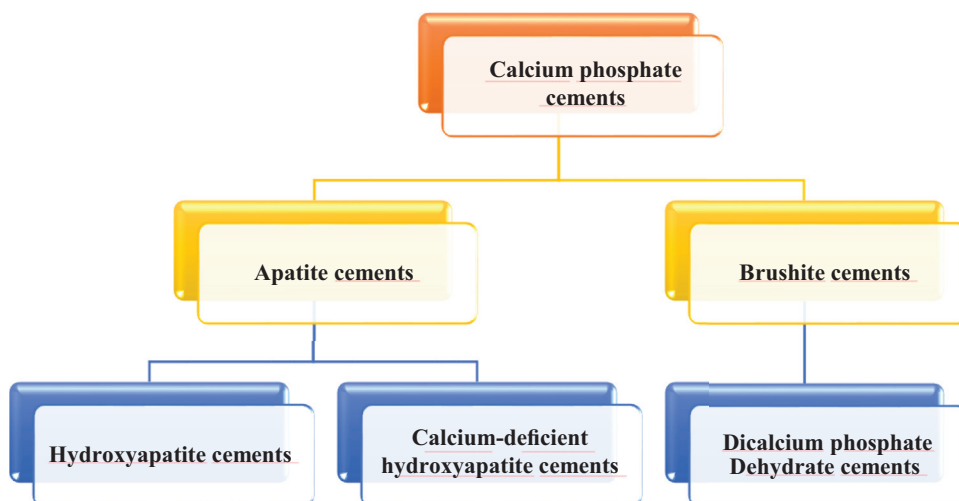


FIGURE 17.3 Classifications of CPCs. CPCs, Calcium phosphate cements.

apatite type of CPCs. Dicalcium phosphate dehydrate (DCPD) is the best known brushite type of CPCs.

An aqueous solution is added to one or more solid COP powder to produce CPCs (Fig. 17.4). These COPs can be in the amorphous and/or crystalline state. Various aqueous solutions such as distilled water [11], sodium citrate [12], sodium hydroxide solution [13], chitosan (98% deacetylated) and glucose [14], polyacrylic acid (50 wt.%) [11], disodium hydrogen phosphate [15,16], revised simulated body fluid (SBF) (rSBF) [17], malic acid (30 wt.%) [18], a solution of citric acid (35 wt.%) [11], a solution of phosphoric and sulfuric acids [19], and sodium polysilicate solution [20] can be used to produce CPCs. Mixing of these materials provides a paste that is malleable and has self-setting characteristics. Due

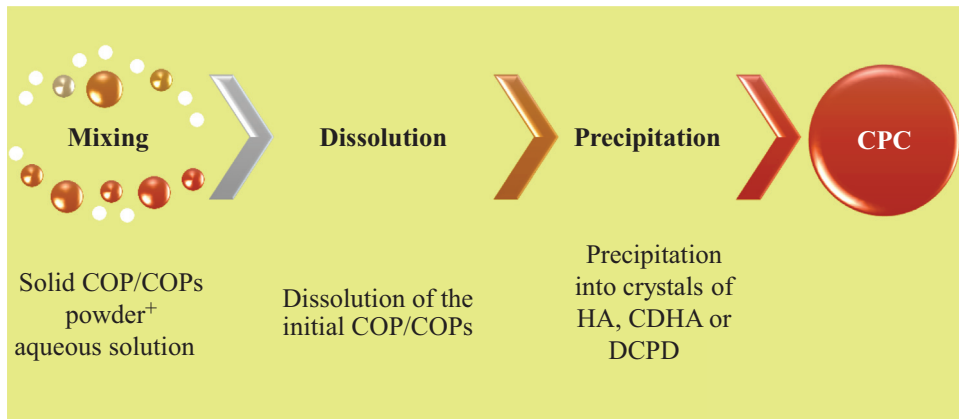


FIGURE 17.4 Basic flowchart for the processing of CPC. CPC, Calcium phosphate cement.

to these properties, such pastes provide an easy manipulation and can be readily modeled into the target bone defect structure or, within a short period of time, can be simply injected into the context of a minimally invasive surgery [21,22]. A relatively less soluble CP phase is formed in the system via a dissolution and reprecipitation mechanism during the setting step [21].

The self-setting COP cements were discovered in the early 1980s and a vast number of new CPC formulations are still being developed today to satisfy the desires of various biological applications. The composite approach has become very popular recently to enhance the properties of the CPCs; and reinforcements such as particles [23,24], and whiskers, fibers [25,26], or microspheres [27,28] are used to overcome the limitations of these materials. Depending on the application area, inorganic or organic-based reinforcements are used. Loading polymers into CPCs is one of these strategies to improve the characteristics of these cements [29]. Synthetic polymers such as poly(lactide-co-glycolide) (PLGA) [30–32], poly(ethylene glycol) [33,34], poly(vinyl alcohol) (PVA) [35], poly(acrylic acid) [36], and natural polymers, such as, cellulose [37,38], gelatin [39], alginate [40], chitosan [41], and chitosan–alginate complex [42] are investigated to enhance the performance of the CPCs. Recently, fibers such as PVA [43–45], polylactic acid [46], and carbon [25] have been investigated as a reinforcement to improve mechanical properties of the CPCs. CPCs consist either fully or essentially of COPs and a list of commonly used COPs and their Ca/P ratio are given in Table 17.2.

During the clinical applications, setting, and hardening characteristics of the CPCs play a crucial role. Microstructure development of the CPC and chemical formulation significantly affects the setting behavior of the material as well as the mechanical performance and degradation rate of CPCs [2,47]. Besides setting and hardening characteristics, many vital properties such as *in vivo* resorption velocity, solubility, compressive strength, and porosity are significantly affected by the synthesis and processing conditions of CPCs. Effective parameters on the vital characteristics of CPCs are given in Table 17.3.

TABLE 17.2 Chemical composition and Ca/P ratio of some calcium orthophosphates.

Name	Abbreviation	Chemical formula	Ca/P
Amorphous calcium phosphate	ACP	$\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$ $n = 3-4.5$ and $15\%-20\% \text{ H}_2\text{O}$	1.2–2.2
Calcium-deficient hydroxyapatite (also known as precipitated HA)	CDHA	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ ($0 < x < 1$)	1.5–1.67
Dicalcium phosphate anhydrous, mineral monetite	DCPA	CaHPO_4	1.0
Dicalcium phosphate dihydrate, mineral brushite	DCPD	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	1.0
Fluorapatite	FA	$\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$	1.67
Hydroxyapatite	HA	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	1.67
Monocalcium phosphate monohydrate	MCPM	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	0.5
Monocalcium phosphate anhydrous	MCPA	$\text{Ca}(\text{H}_2\text{PO}_4)_2$	0.5
Octacalcium phosphate	OCP	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	1.33
Tetracalcium phosphate, mineral hilgenstockite	TTCP	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	2.0
α -Tricalcium phosphate	α -TCP	$\alpha\text{-Ca}_3(\text{PO}_4)_2$	1.5
β -Tricalcium phosphate	β -TCP	$\beta\text{-Ca}_3(\text{PO}_4)_2$	1.5

TABLE 17.3 Essential parameters effective on the characteristics of calcium phosphate cements (CPCs).

Property	Effective parameters
Solubility	Solubility of CPs in physiological conditions depend on the chemical composition and applied fabrication conditions
Bioactivity	Crystallinity, phase content, ratio of Ca/P, and purity are effective parameters on the bioactivity of CPCs
Degradation behavior	Crystallinity, ratio of Ca/P, phase content and purity are generally affective on the features of degradation phenomena
Setting behavior	Microstructure is very effective on the setting behavior of CPCs
Hardening characteristics	Synthesis and processing conditions of CPCs affect hardening characteristics
Compressive strength	Porosity content (pore morphology, size, and distribution), chemical and phase composition
Density	Phase content, microstructure, and porosity

17.2 General characteristics of calcium phosphate cement

CPCs have more and more been employed as bone replacements since their original formula in the 1980s. This chapter gives an overview of the chemical, kinetic characteristics of bone replacement (setting time, cohesiveness, and injectability) of CPCs, with focus on

the mechanical characteristics of the bone. Many parameters, such as particle size, concrete reactant structure, and additives can be adapted for monitoring and mechanical performance control of the CPC settings system in processing [48]. A broad variety of synthetic materials were suggested and created as bone replacements, including metals [49], ceramics [50,51], polymers [52,53], and cements [6,54,55]. Their outstanding biological conduct has given CPC great importance [48]. Among them, the outstanding biological conduct of CPC has been very attractive [56,57]. In the 1980s, Brown and Chow first created the CPCs. Since that time, many CPCs have been researched and commercially available with different compositions. In CPC production, a two-phase chemical reaction occurs and the mixture hardens into a solid mass progressively. The strong stage consists of one or more compounds of CaP. Water or a solution containing CP is applied as a liquid and may include chitosan [58], alginate [59,60], hyaluronate [61,62], gelatine [63,64], sulfate of chondroitin [65,66], succinate [66], or citric acid [67], which allows for original CaP compounds to dissolve until the solution has been oversaturated inducing the reprecipitation of crystals. There are currently only two possible end products for the CPC response despite various CPC formulations: brushite (DCPD), or apatite (HA or CDHA) [40]. There are also several options for the CPC response. In addition, two kinds of chemical reactions are mainly acquired from these two final goods: hydrolysis and acid-based [22,50,57]. The main distinction is their solubility: 1–2 magnitudes of brushite is soluble in 1–2 orders more than apatite at a physiological pH [68]. Their solution is important. As brushite is, however, a metastable stage, brushite can be converted *in vitro* to apatite [40]. The primary benefits of CPCs are, in relation to their outstanding biological behavior, that they can be injected and can harden *in vitro* at body temperature [69]. A mixture of solid and liquid phases forms a slimy paste that can be easily shaped or injected into defective surfaces. This characteristic of CPCs bypasses invasive procedures and provides a firm adjustment to the surrounding bone even for not evenly shaped cavities. *In vivo* acrylic cements [e.g., poly(methyl-methacrylate) (PMMA)], which have broad applications in arthroplasty and vertebroplasty, can also be found to have the characteristics of injectability and hardening [70]. However, PMMA has an extremely exothermic hardening method (also referred to as polymerization) causing necrosis of the surrounding tissue [71]. The hardening of CPCs, by comparison, is only mildly exothermic if not important for biomedical applications, as well as incorporating various biological molecules and medications [50,69,72–74]. The fact that the CPCs are intrinsically microporous [50] is another significant characteristic. After hardening of CPCs, and/or intergranular spaces, the micropores are left with extra aqueous solutions with a pore size in the micrometer range. These micropores are helpful when biologic liquids are impregnated and resorbed in CPCs and replaced by bones. However, in order to favor bone colonization in the implant, macropores with at least ten meters of CPC would also be desirable, thus speeding up the overall process of replacing CPC with bone, as was the case with bioceramics in CaP [75,76]. The pores do not only contribute to the abovementioned biological behavior in CPC but also enhance the surface area for CPCs, which can be used to react, thus improving their capacity to load growth factors or drugs. Whilst CPCs appear to be very promising to regenerate bones, it is commonly recognized that some important problems still need to be dealt with in order to fulfill clinical needs [48]. In particular, the fluid, strong phase separation of CPCs without any additives is usually poorly injectable [77,78]. In addition, CPC pastes tend to

disintegrate as a result of their fragile cohesion after early contact with blood or biological fluids [79]. Another major challenge is the overall bad mechanical characteristics of CPCs, not just in relation to commonly researched resistance but particularly as regards to toughness, fragility and reliability, rarely noted, which limit their implementation to locations with low loads [80]. In recent decades, a significant effort and numerous researches have been undertaken in order to explore and understand, with various successes, the mechanisms under the aforesaid issues in CPCs. It aims to provide an overview of the chemical and kinetic characteristics of CPCs for bone replacement and to determine the most important accomplishments, with a focus on their mechanical characteristics [48].

17.3 Chemistry and handling

The response of cement settings may be the most significant element in CPCs because it not only directly regulates the durability of cement hardening moment and other settling features but also determines the nature and therefore the physical and biological characteristics of cement hardening [48]. Currently, countless combinations of compounds containing calcium and phosphate are found in CPCs. The chemistry of setting reactions in these cement structures is nevertheless comparable, and the solubility conduct of the compounds concerned can be clarified and understood by assessment [54,81]. Diffusion and recipients are primarily involved in the chemical process during the setting reaction [82]. The starter powders release calcium and phosphate ions during dissolution, which generates supersaturation. Once the ionic concentration reaches a critical value, the fresh stage is nucleated around the powder particles in general. The fresh stage then begins to grow with the continued dissolution of the reagents [69]. The final structure of the precipitation is dependent on the comparative stability of the different CP components in the scheme and can be predicted by using the diagram on the solubility stage, describing the development of solubility—in the form of the logarithm of the complete calcium concentration (or phosphate)—in terms of the pH. Specifically, they dissolve into a more stable (less soluble) phases of CP [83]. Since apathetic is $\text{pH} > 4.2$ (37°C) and brushite is the most stable CP at $\text{pH} < 4.2$ (37°C), that explains why the CPC response is only two primary end products, despite various CPC formulations [83]. The thermodynamic behavior of the CPCs can be predicted by diagrams for solubility phases, but the observed setting or hardening behavior cannot always be explained and cinematics should also be taken into consideration. Understanding the processes that regulate the setting process of CPCs will assist in obtaining a full understanding of its kinetics and regulate their microstructure, which determines its applications for distinct reasons. At present, CPC substitutes are mostly based on the hydrolysis of the α -tricalcium phosphate (α -TCP) powder which is used in majority of commercial cement formulations. Many studies focus on the impact of [84–86] particulate size temperature, and multiple components [87]. Many of the techniques used in the development of the setting response were: X-ray diffraction (XRD), isothermal calorimetry, resistance measurement, nuclear magnetic resonance (NMR), impedance spectroscopic, and attenuated complete reflection—Fourier transforming surface spectroscopy [84,86,88,89]. The magnitude of α -TCP converting to CDHA was assessed by Fernandez et al. [90] by using the height from several chosen XRD peaks, assuming a quasi-constant

proportion from peak to the maximum. Ginebra et al. [15] used an internal normal procedure for calculating the comparative quantities of various stages in the sample. Both authors found that the extent to which α -TCP can be converted to CDHA could be exponentially adapted depending on the durability of the process. In addition, organizations of these two writers also examined and outlined with linear equations the development of compressive resistance of CPCs. In addition, the compressive strength and converter range were linearly correlated [15]. Ginebra et al. [91] further created a relation between the reaction progressive depth and the reaction time and suggested two speed limiting processes for α -TCP kinetics. In comparison, the first process, "Reactant Surface" checks for the response during the original phase; after 16 hours, the remaining response is controlled by the other system, "propagation by the hydrated layer." Ginebra et al. [92] examined the impact on the kinetics of the configuration response of various particle dimensions of α -TCP by merging XRD and vigor information. Fine particles showed that hydrolysis is much quicker than coarse particles. The fact that a greater region accelerates the method of dissolution could be readily described. Despite its simplicity, XRD has restricted quantitative stage analytical capacity, particularly for poorly crystallized or amorphous materials, and thus a more precise way to understand the hydrolysis frequency and reaction mechanisms is needed. For the monitoring of concrete setting response, AC impedance spectroscopy was used. AC impedance spectroscopy is an efficient technique to investigate hysterization without interruption by representing the growth of the microstructures through ongoing identification of the AC impedance [93], as opposed to the abovementioned mechanical (measuring force) and compositional (XRD or NMR), which generally monitor cement environment intermittently. Isothermal calorimetry is a method frequently used for the examination of kinetic reactions. Durucan and Brown [85] examined the kinetics of CPC by using isothermal calorimetry, based on α -TCP of three particle dimensions. Their results correspond to the XRD statement that the setting response indicates that the particle size is strongly dependent. The writers also suggested that the α -TCP hydrolysis response, compared to the model recommended by Ginebra et al. [91], be regulated originally by a ground system, and consequently by a nucleation and development system. This model was a little distinct. The reactivity of three amorphous α -TCP nanoparticles synthesized by a spray flame, and that of micro α -TCP, was evaluated by Brunner et al. [84]. These nanoparticles showed a pronounced rise in reactivity, with the complete energy discharge being continuous during hardening. In summary, the kinetics of α -TCP environment is influenced by many variables, including particulate size, crystallinity, heat, structure, or even physical alteration of the reactant surface [94]. In addition to its outstanding conduct, the two major benefits of CPCs as bone replacements are being injected and self-setting *in vitro* at body temperature. CPCs usually have a comparatively lengthy setup, bad shock injection, and bad cohesion, but without any improvements [95,96]. A lengthy setting period CPC paste can trigger several difficulties. For example, if the CPC was unable to set up and disintegrate, a severe inflammatory response occurred [97]. A weakly cohesive CPC can also create major challenges. A study [98] showed, for instance, that when in touch with sperm, a CPC for vertebroplasty induced blood clotting that was triggered by blood-to-solid particle interfacial responses. All of the above disadvantages are regarded as difficulties for the wide-ranging implementation of CPCs. For that purpose, the above managerial properties will be detailed and in the following subsections, the

methods used to improve them are introduced. Mechanical and chemical characteristics are the two most significant characteristics of metals in the majority of operational apps. The development of a new biomaterial should, therefore, take both the mechanical properties and reactivity into account. The first is that the mechanical characteristics of a substance are based on its microstructure from a material science perspective. A range of microstructural characteristics is achieved through various manufacturing paths and handling parameters. Therefore, the connection entry manufacturing and mechanical characteristics are essential to the microstructure.. In order to connect mechanical features immediately with manufacturing without having to do both with microstructure, it will be totally impractical and pointless to understand the theory and to effectively design specific features. The following subsections will, therefore, be considered for microstructure—mechanical characteristics. CPCs are created by a dissolution—reprecipitation method at space or body temperature, in contrast to bioceramics that need to be sintered at elevated temperatures. A network of apatite crystals, accountable for the mechanical characteristics of CPCs, is created through this method. Over time, the dense network and the apatite crystals continue to develop until the cement has its mechanical characteristics to the maximum. Most mechanical characteristics have been evaluated by compressive or tensile charging, providing the compressive or tensile strength values and sometimes the relevant elastic modular. It is worth noting that in most trials, alternative (indirect) tensile testing methods for measuring flexural resistance or diameter tensile resistance (DTS) were employed because of the difficulty to directly measure the tensile resistance of CPCs, although the bending test generally gives higher values and the DTS test gives lower values compared with true tensile resistance [99]. Conversely, there have been few reports of toughness and reliability studies [100,101]. The mechanical characteristics of CPCs are highly dependent on their microstructural characteristics such as porosity, quantity, shape, morphology, and the allocation of apatite-shaped crystals. Moreover, all technological variables engaged in the manufacture of CPCs relate to these microstructural characteristics. It is therefore conceived that the comparative sizes of the reagents in the blend, as accelerators or retarders, L/P, the stress during sample preparing, and the aging condition will influence their mechanical characteristics, all considerations such as the technical structure of the concrete will influence the mechanical compounds. Porosity is one of the primary parameters affecting the biological activity and the mechanical characteristics of the biomaterials. As stated earlier, CPCs have an intrinsic microporous characteristic. The L/P ratio depends significantly on the microporosity of CPCs, which typically vary between 30% and 55%: the higher the L/P ratio, the higher the microporosity [102]. Macropores are also desirable to enable the growth of bones within the CPC, improving its biosorption and accelerating the replacing of bones by new ones, as are intrinsic micropores that allow for the impregnation of biological fluid. Porogen leaching, macro forces after setting [103–106] and air foaming, generate macro forces before setting, and they are the most prevalent methods for the creation of macro forces in CPCs. Neither technique is, however, exempt from inconvenience [48]. It is common to accept that both kinds of pores are harmful to force despite the distinct tasks of micropores and macropores in biological activity. It should also be noted that the specimen test circumstances impact the compressive strength values measured in addition to the impact of the pore itself. In addition, given the experimental

circumstances, it is worth noting that the CPCs' tensile or folding power is generally significantly less than their compressive strength comparable to most brittle metals. In fact, fractures occur through crack propagation due to existing faults, such as microcracks, and a crack in strain is usually simpler than compressive to propagate [107].

17.4 Biological evaluation of calcium phosphate cements

The composition and crystal structure of CPCs have a similarity to the teeth and bone minerals; therefore, it shows some common properties of them such as biodegradability, bioactivity, osteoconductivity, and osteoinductivity (Fig. 17.5) [108–110].

17.5 Biodegradation of calcium phosphate cements

Biodegradation (bioresorption) refers to the process of removal of a material from the body by cellular activity. The patient should be hospitalized and operated during the removal of the implanted material, and the biodegradable material eliminates this distress experience. A gradual replacement of the material occurs by generating space for new cells to form functional tissues. Biodegradation takes place in three steps; in the first step, the material is divided into simpler composition by the cells, a physical breakdown and chemical dissolution take place as the second and the third step, respectively. The degradation of the CPCs is based on either passive or active resorption. Passive resorption is a simple chemical dissolution that accompanies the loss of structure of cements. But, active resorption is a cellular process and an acidic environment is created by osteoclastic cells and solubility of the CPCs is achieved [32,111–114]. Some parameters affect the biodegradation of CaPs; composition, particle size, crystal structure, and pore characteristics. Studies revealed that with increasing Ca/P ratio, the biodegradability of CaP decreased. Among the CP ceramics, the highest degradation the observed in amorphous CP whose Ca/P ratio is at least 0.67 [115]. Basic CPCs (i.e., apatite) ability its to resorb slower than acidic CPCs

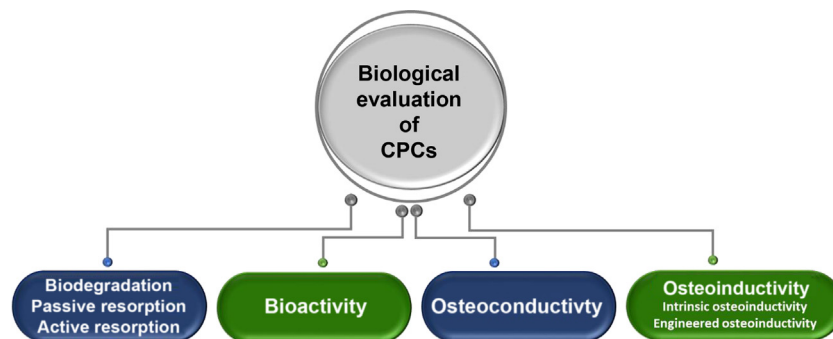


FIGURE 17.5 Biological evaluation of CPCs. CPCs, Calcium phosphate cements. Source: Adapted from LeGeros RZ. Calcium phosphate-based osteoinductive materials. *Chem Rev* 2008;108(11):4742–53.

(i.e., brushite/monetite) *in vivo*. Apatite cement is more stable and may exist in body in years. On the other side, brushite and monetite are highly soluble, the within a few months they resorb *in vivo* [116]. Besides Ca/P ratio, incorporation of some elements or compounds into CPC structure alters the biodegradability. Whereas the solubility of β -TCPs is reduced by the addition of Mg^{+2} or Zn^{+2} [108].

Bohner and Gbureck investigated the relation of particle size-setting behavior of β -TCPs by using calorimeter. Increasing the milling time of β -TCPs improved the reactivity of the cement, the main reaction peak formed at an earlier time point and became larger [117].

Macroporosity also favors the degradation and replacement by bone tissue of CPC. Cell penetration and fluid flow throughout the material is formed by macroporosity and both active and passive degradation is being induced. There are various methods to obtain macroporosity in CPCs; addition of inclusion that can be dissolved in water, foaming agents, and biodegradable polymeric microspheres as porogens. Saccharides, chitosan, sodium chloride are examples for water-soluble additives that form macroporosity in CPCs. These additives are dissolved either before implantation or gradually resorbed *in vivo*. Porosity is produced by incorporating gas into a slurry and then set in order to keep the air bubbles stable by foaming agents. In CPCs typical foaming agents are hydrogen peroxide and carbon dioxide. Foaming is a cheap, nontoxic method (there is no risk of formation toxic gases during the use of implant) and provides various porosity levels and pore size ranges in CPCs [69]. The last method is the inclusion of biodegradable polymers (PLGA) microspheres as pore forming agent. Hollow microspheres are made of biodegradable polymers form macroporosity during the degradation of microspheres. Also, o biodegradable polymers can accelerate the degradation of CPC matrices [32]. The patient related parameters like age, gender, life, etc. can affect the resorption rate [69].

17.6 Bioactivity of calcium phosphate cements

Bioactive material has the ability to form a direct bond to hard bone tissue without the formation of fibrous tissue. In a biological fluid a carbonated HA (HCA) layer is formed if the material shows bioactive property. Effective bonding between bioactive material and human bone occurs via this layer. When cement paste is implanted, bioactivity provides a stable connection between the defect and the implant. Subsequently, resorption of CPCs occurs by two ways as mentioned in the previous section. Although the *in vitro* bioactivity of the CPCs has been tested by immersion in SBF, some conflicts have existed on the prediction of *in vivo* bioactivity of a material via SBF. The results may be misleading, therefore *in vivo* implantation in a bone defect is more reliable. *In vitro* HCA formation on CP-based materials in SBF is driven by dissolution and precipitation processes; calcium and phosphate ions from the SBF are being received [69,108,116–120].

By altering the composition, the bioactivity of CPCs can be improved. Si^{4+} , Mg^{2+} , Sr^{2+} , and different cations were added into the CPCs system to increase the bioactivity of the material. Various proteins, especially growth factors, are responsible for further promoting of new bone formation. Another factor on bioactivity is the porosity, and the pore size less than 1 μm is responsible for the bioactivity [69,119].

17.7 Osteoconductivity of calcium phosphate cements

When a material stimulates the ingrowth of new bone into a surface or a part in the material, it has osteoconductive property. Bone growth on the surface of the material or inside the pores or channels is observed for osteoconductive materials. CPCs are accepted as osteoconductive because HCA layer is responsible for the attachment, proliferation and differentiation, and it leads matrix production and biomineralization. In Fig. 17.6, the growth of bone tissue around β -TCMP (Mg-substituted tricalcium phosphate) proves the osteoconductive behavior of CPCs [108,110,116].

One of the important parameters in osteoconductivity is porosity. The ideal porosity is 60%–80% with interconnected nature. The pore size varies between 150 and 500 μm . These pore characteristics provide cell penetration, nutrient exchange, and waste removal. Another important parameter is chemical composition [108,116,119]. Zhao et al. [121] showed the influence of the pore size on osteoconductivity. CPC scaffolds were with the same porosity, however three different pore sizes (200–300, 300–450, and 450–600 μm) were produced and filled into radius bone of a rabbit. The osteoconductivity was assessed after 4, 12, and 24 weeks by alkaline phosphatase (ALP), histological assessment, and mechanical testing. They concluded that scaffold with the smallest pore size promoted bone formation in 4 and 12 weeks. Higher ALP values, formation of some granulation tissues, and higher compressive strength value (110 MPa) were obtained for a scaffold with 200–300 μm pore size. At the early stage of osteoconductivity, migration and activity of osteoblasts were provided by a smaller pore size. Besides, higher surface area favored migration and adhesion of cells [121].

Like in biodegradation and bioactivity of CPCs, chemical composition also affects the osteoconduction. Some ions contribute to osteoconductivity such as Si and Sr. When these ions were introduced into CPCs structure, they increased proliferation and accelerated degradation was obtained. Hence osteoconductivity accelerated. Julien et al. [122] studied the doping of amorphous CPC containing ions like Mg, Zn, or F. Cement with F had larger crystallites precipitated, was this showed was apatite formation was completed. Also, higher compressive strength was obtained compared to CPCs doped with Mg or Zn [122].

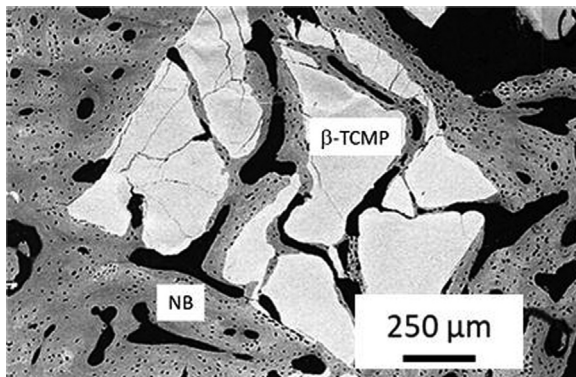


FIGURE 17.6 NB formation around and Mg-substituted tricalcium phosphate (β -TCMP) after implantation [108]. NB, New bone.

17.8 Osteoinductivity of calcium phosphate cements

Osteoinduction refers to the stimulation of progenitor cells to develop osteoblastic cell lineage. If the material performs osteoinductive property, it promotes osteogenic cell differentiation [108]. Osteoinduction is classified into two groups: active and passive osteoinduction. BMP and other growth factors are used during active osteoinduction. When the material induces osteogenic cell differentiation via its specific properties (microstructure, etc.), it shows passive osteoinduction. It is accepted that CPCs are osteoconductive but not osteoinductive. Some studies showed that CPCs can form bone in nonbone-forming sites *in vivo* without osteogenic factors. Since this osteoinductivity experienced some sort of CPCs, it is called intrinsic osteoinductivity. Composition, topography, surface area, porosity, etc. are effective parameters for intrinsic osteoinductivity [108,119]. Heughebaert et al. [123] prepared the implant with 97% HA and 3% β -TCP and placed in two nonbony sites. The study concluded that HA-based implant and inorganic part of bone had important similarities such as composition, phase content, etc. [123]. Other CPC compositions that have osteoinductivity are basic CP, DCPD, dicalcium phosphate, carbonated apatite, and octocalcium phosphate [109]. Higher surface area produces sites for entrapment and concentration of BMPs and osteoprogenitor cells [32,69,108–117,119]. Yuan et al. [124] investigated osteoinductivity of nine different materials including eight sorts of CPs (two different cements) and TiO_2 . Microporosity was introduced into all materials (except one sort of HA) and they were placed into muscles of dogs. Results showed that three materials did not display bone formation; one including HA without micropore [124].

Engineered osteoinductivity is obtained by adsorption of osteogenic factors that can enhance cell adhesion, differentiation, matrix formation, and biomineralization apart from the properties of materials. The factors combined are osteoprogenitor cells, bone growth factors, and bioactive proteins [108,125]. Li et al. [125] investigated the osteogenetic capacity of three materials: CPC, a biocomposite (fabricated from mesoporous bioactive glasses and CPCs,) and biocomposite combined with protein. The used protein was recombinant human bone morphogenetic protein-2. *In vitro* (bone marrow stromal cells) and *in vivo* (rabbit radius defect model) studies were conducted for three materials. Strong osteoinductivity was observed for protein-loaded biocomposite, the rate of new bone formation and mineralization is the highest at the early stage among all three materials. Also, more osteoid depositions and better biodegradability were obtained for protein-loaded composite [125].

17.9 Cellular response to calcium phosphate cements

Biomaterials used in biomedical apps are exceptional in their capacity to form biomimetic materials and have different features [126]. These substrates are the most complicated topographically and generally produce a favorable cellular response for the recovery of tissues. Their structure can also provide the resorbing implant with an efficient chemical gradient inducing required cellular response which contributes to fast healing and recovery [127]. Due to the alkaline microenvironment wealthy in calcium and phosphorus

particles in a proportion comparable to the extracellular tissue structure, exceptional bioactivity of apatite forming CPCs is achieved [126]. Furthermore, the intrinsic microporosity of these products helps the discharge of drugs and biomolecules that have been shown to guide cell activity so that wound healing and reshaping can be achieved in near proximity to the environment [128]. In addition to osteoconductivity, biocompatibility is the most significant necessity for a bone replacement electrode product. The desired capacity of the biomaterial is to execute its requested medical purpose without producing unwanted local or environmental impacts in the receiver or beneficiary of the treatment, but to generate and optimize the clinical output of the treatment in the most suitable positive cellular response [129]. Orthopedic implants are intended to degrade the implant in time for unsustainable, short-term touch. Therefore in relation to a suitable positive cellular response, the implant fabric should have a degradability rate in the flesh [126]. Studies on the *in vitro* assessment of microporous CPC showed a greater level of biosorption because of both increased bodily fluid touch and increased cell activity owing to grain breakup [126]. The kinetics of passively resorbed samples, ionic replacements, Ca:P ratios, crystallinity, and pH for cement tissue interfaces depend on their kinetics. Cellular response results in effective resorption; however, this is also associated with passive activity [126]. Cells can be spread over three stages—an original lag phase, during which cells can adjust to the fresh setting and substitutes the ECM that was demolished during trypsinization; a regular stage, during which cell numbers exponentially rise when cells still are an undifferentiated condition; and lastly a plateau phase, during which the cells are in an undifferentiated state [130,131]. An enhanced calcium level of those HAp in the microenvironment may lead to the rapid differentiation and mineralization of cells found in these matrices; this might lead to an enhanced level in intracellular calcium of cells [132]. The MC3T3-E1 cells have calcium receptors stimulated by voltage in the blood membrane, and intracellular activity is controlled by calcium ion density.

17.10 Clinical applications

Increased life expectancy [86] enhanced the need for biomaterials for dental, craniofacial, and orthopedic applications. Besides, due to diseases and traumatic events, an extensive number of patients need to undertake bone grafting operations each year [133]. In the elderly, fractures have recently been perceived to escalate in frequency and severity [134]. More than 6 million fractures occurred in the United States from 1992 to 1994 [135,136], while 7 million people suffered fractures in 1998 [137]. In 1995 musculoskeletal conditions cost the US\$215 billion [136,138]. These numbers are predicted to rise dramatically because of the increasing life expectancy [5,6]. Bone grafting was first created two millennia earlier by the clients themselves (autograft) or by suppliers (allograft). It is the method for removing lacking and harmed bone products [139,140]. At present, an autograft is still regarded since a gold standard as live cells and growth factors are present in the bone extracted from the clients themselves [48]. However, autograft has a number of constraints such as extra operations with related pain and morbidity on the second surgical location as well as the apparent lack of bone references [48]. Modern allografting with donation bone in the periodic bone bank could, however, partially solve restrictions on bone production,

but after sterilization, the biological components of the bone are lost and power is reduced [139,141]. In addition, issues remain concerning the immunological response of the person to the donation bone and transmission of disease; regeneration can be unstable in some instances. In some instances [142] due to the above inconveniences, demand is rising for synthetic replacements free from bone production, inconsistent conditions and diseases [49]. These substitutes can also be applied in conjunction with one's own cells or with recombinant growth factors to enhance and promote the quality of bone regeneration which constitutes the bases of "tissue engineering" [143].

A wide range of synthetic materials, such as metals [49], ceramics [50,51], polymers [52,53], and cement [6,55,83,144–146] have been candidates as bone substitutes [49]. Their outstanding biological conduct also attracted considerable consideration among these CPCs (e.g., biocompatibility, bioactivity, and osteoconductivity) [57,108,147,148]. An undesirable fibrous may be formed by bioinert implants without bone-like CaP mineral, whereas bone-like mineral implants benefit from bonding to the original because the CaP biomimetic mineral offers a favored tissue fixation substratum, it promotes osteoblast phenotype growth and speech [69]. Hence, HAp and other CaP bioceramics are important for tissue repair with their osteoconductivity and bone-bonding ability [70]. The surgeon needs to apply the desired shape to the graft or carve the surgical site around the implant to fit the sintered HAp and other bioceramics into a bone cavity. All these extra applications increase the bone loss, trauma, and surgical time [69]. The contours of defect structures can closely be adapted by reforming CPCs and positioning them. CPC is composed of a mixture of tetracalcium phosphate [TTCP: $\text{Ca}_4(\text{PO}_4)_2\text{O}$] and dicalcium phosphate anhydrous (DCPA: CaHPO_4). Another important feature of CPCs is that they are intrinsically microporous [48,72]. Micropores of the intrinsically microporotic CPCs are a byproduct of extra aqueous solution after hardening of CPCs and/or due to intergranular spaces. The pore size may vary in the range of micrometers. Such micropores are useful for the penetration of biological fluids into CPCs and are useful in resorption and replacement of CPCs by bone [72]. Macropores should also be created to speed up the substitution process of CPCs by bone and promote bone colonization in the implant [75,76]. Not only do pores play a critical role in the above biological activities of CPCs, but they also raise the bottom of CPCs accessible for the response, improving their capacity to handle growth factors and the supply of drugs, thus creating cells excellent applicants for the development of bone tissue (Fig. 17.7).

CPC can be blended with a fluid to create a pulp capable of closely matching the surrounding bone to form HAp, even for complex osseous abnormalities and in situ self-hardening at space or body temperature [64]. CaP mineral implants benefit from host bone-bonding as CaP is a substratum for osteoblast phenotypes [65,66] cell bonding, proliferation, and expression. Thus because of its good moldability, self-hardening in situ and excellent osteoconductiveness, CPC is a highly promising composite in bone engineering. A popular element used in CPCs is α -TCP. Studies [67–70] show that the TCPs can be osteoconductive for fast bone inclusion and that they are absorbed at the optimum pace following fresh bone formation either alone or in conjunction with other CPs [1]. Since CPC was first suggested two decades ago [9], its characteristics and efficiency have been widely enhanced. Researches have been carried out with the combination of resorbable fibers [71] to enhance the mechanical characteristics of the CPC. One such fiber is the

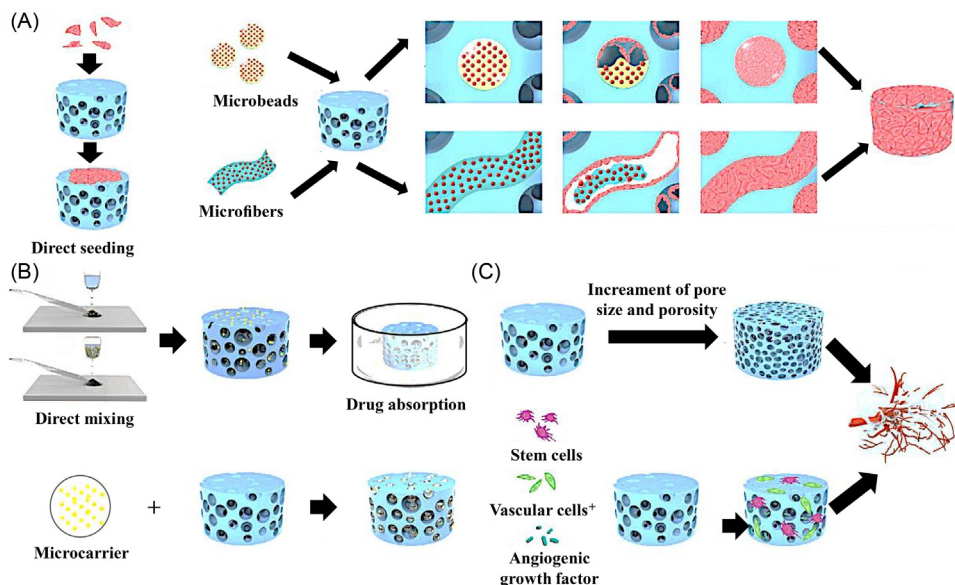


FIGURE 17.7 Schematic outline of the various CPC categories described in this chapter and their major biological characteristics. (A) Cell delivery, (B) drug delivery, and (C) vascularized CPC scaffolds [119]. CPC, Calcium phosphate cement.

clinical suture of silk fibroin (SF) which has been in use for centuries. SF is an outstanding mixture of qualities such as lightness, high strength, notable tightness, and elasticity [72]. Subsequent studies [73,74] have demonstrated SF biocompatibility and are capable of encouraging bones and maturation in a significant way in order to enhance body adhesion and development in several ordinary adult human cell types, including osteoblasts and fibroblasts. Mesenchymal stem cells (MSCs) are multipotent cells that can be differentiated into bones, cartilage, fat, muscle, glia, and neurons. In addition, even when expanded *in vitro* for long periods of time they can continue their differentiating potential [75]. Due to their outstanding characteristics, MSCs were commonly recognized as an appealing cell source for tissue technique. MSCs were not cultivated in combination with the osteogenic supplements (β -glycerophosphate, ascorbic acid and dexamethasone) on a powerful CPC–SF scaffold yet and were driven to distinguish into the osteogenic lines [76]. The goals of the research were (1) to create high-force osteoinductive CPCs, that is, cement compound (SF/OS/ β -TCP); (2) to explore MSCs interactions in this composite and MSCs proliferation; and (3) to explore osteogenic differentiation of MSCs on this composite [76]. The primary benefits of CPCs are the capacity to ingest and *in vitro* adhere to body temperature, in relation to their outstanding biological behavior [57]. The CPCs form a viscous pulp when the solid–liquid phases are mixed. This pulp can be easily manipulated and shaped and even can be injected in a defective area. This characteristic of the composite is useful in avoiding invasive surgical operations and provide an intimate adjustment to the surrounding bone even for irregular surfaces (Fig. 17.8) [149]. The characteristics of being

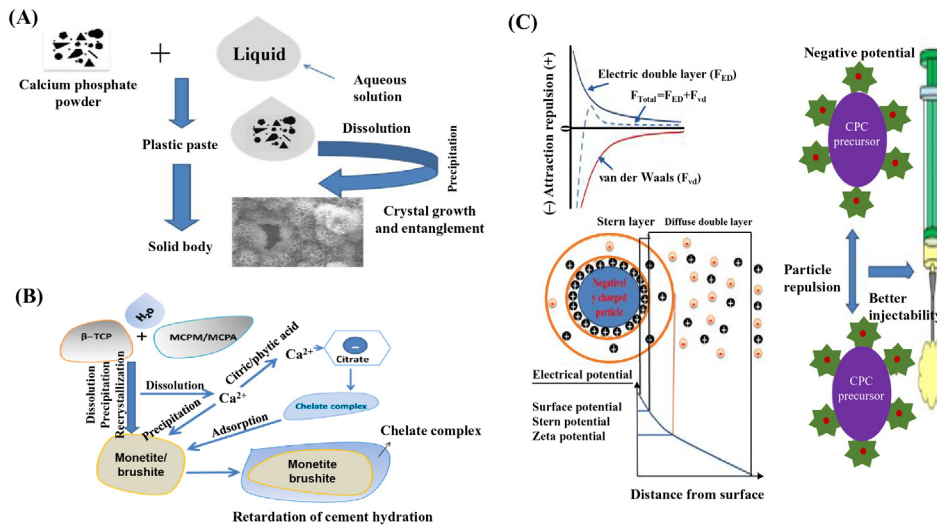


FIGURE 17.8 (A) Setting mechanism of CPCs. (B) Effect of polycarboxylic acid in retarding setting behavior of CPC. (C) Effect of zeta potential of CPC slurry in particle deagglomeration and improving its injectability. CPC, Calcium phosphate cement.

injectable and of hardening *in vivo* are common in acrylic bone cement (e.g., PMMA). Although PMMA is widely applied in arthroplasty fixation and vertebroplasty, the hardening process (also called polymerization) of the material is highly exothermic and has complications such as necrosis in the surrounding tissue [70]. The hardening of CPCs, by comparison, is, if not exothermic, only mildly essential for biomedical apps and the inclusion of various biomolecules and medications [48,57,73]. The CPC dust can be blended to create a glue that may be carved during the operation to match flaws in difficult cells with an aquatic fluid. The powder hardens itself to shape absorbing HA [19]. Excellent osteoconductive and bone replacement capability make CPCs highly promising composites in a wide range of clinical applications [24–26].

Since 1996, CPC has been available for clinical use as a Food and Drug Administration approved composite to repair craniofacial defects in humans [25]. However, some crucial issues still need to be solved to satisfy clinical requirements [3]. One such challenge is the poor injectability capacity of CPCs in the absence of additives due to the liquid–solid phase separation [33]. The fragility and poor resistance also restrict the usage of CPC to noncarrying regions. Another shortcoming of the CPC is the delayed integration of the composite with the neighboring bones due to its nonmacroporotic structure. The use of CPC was mainly reserved for the regeneration of nonstress-bearing bone while significant stress-bearing applications are not included in indications [24], and none of the indications include significant stress-bearing applications. In periodontal restoration, premature fractures and subsequent exfoliation of the CPC implants have led to dental flexibility. An improved CPC also involves a rise of the nose and maxillary membrane because CPC can be formed according to the form and placed as a scaffold for the development of the body. However, these implants are prone to momentary prosthesis early loading and must be

stable in flexure processes. Major maxillary or mandible reconstructions require an enhanced fracture resistance and a rapidly conducting molded implant after trauma or tumor resection to support metal dental implants or increase in implant deficiency sites. The use of extremely biocompatible composite, like enhanced CPCs with stronger fractures with rapid bone regeneration through macropores and the supply of osteogenic cells and growth factors could be used for all these dental and craniofacial applications. Moldable/injectable, mechanically strong and in situ—hardening CPC composite scaffolds have been formulated using absorbable fibers, biopolymer chitosan, and mannitol pyrogens. The relation between texture, fiber, and porosity characteristics has been created in these composites, which significantly improves injectability. In the nanoapatite matrix, macropores produced the composites better suited to cell infiltration. Fresh composites such as CPCs, similar to apatite and CPs found in mammalian bones, are noncytotoxic and they encourage osteoblasts to adhere, propagate and to proliferate. Cells can penetrate the macropores, create cell–cell connections, and hold the micro apatite pores (Fig. 17.9). For cell shipment, cell–CPC–chitosan–mesh builds have also been developed. Three features are provided by alginate hydrogel crystals: (1) They act as a vehicle to deliver cells and nutrients into CPC–chitosan and CPC–chitosan–mesh composites, (2) they protect cells from changes to the environment during cement, (3) by subsequently decaying the hydrogel beads, the powder-to-liquid ratio and chitosan content can be regulated as a protein discharge from CPC to be applicable [150]. The relatively strong and osteoconductive CPC composites may be efficient means to encourage bone regeneration for osteoinductive development, for delivery of antibiotics and other molecules. Growth factors can also be incorporated

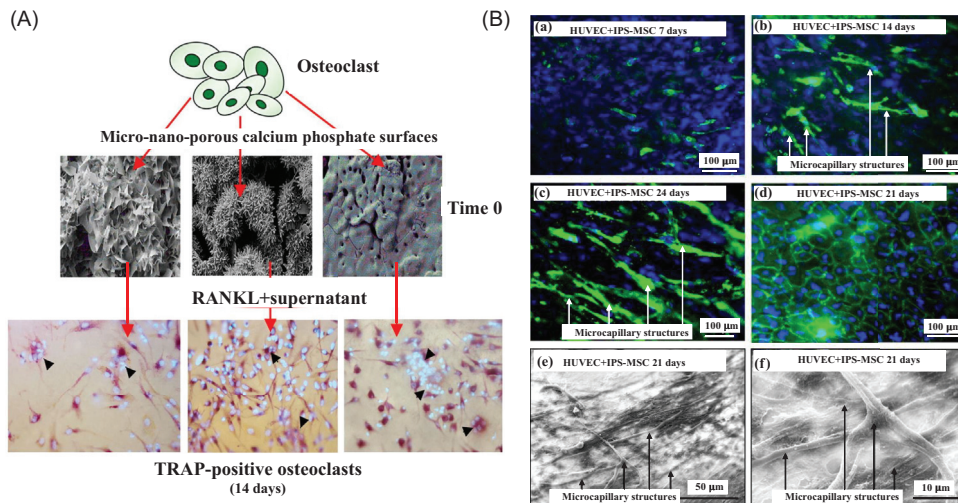


FIGURE 17.9 (A) Human osteoclast and CaP surface. (B) Patterns of microcapillary-like structures formed by Human umbilical vein endothelial cells (HUVECs) and Induced pluripotent stem cells-Mesenchymal stem cells cocultured on CPC scaffolds. HUVECs were identified with an endothelial marker by immunostaining (a–d). Representative scanning electron microscopy images of microcapillary-like structures formed by the co-culture system (e, f). CPCs, Calcium phosphate cements.

into bone grafts as a highly beneficial tool in tissue engineering [151–154]. Various orthopedic, dental, and craniofacial reconstructions of defects are other possible applications [137]. The cell growth factor paste, for example, can be used to fill bulk cavities into bones with the meshes and the fibers that cover the bones and provide the required strength. Minimally invasive operations such as the fixation of in loc fracture and percutaneous vertebroplasty for osteoporotic bone lesions, which are at risk of fracture are other desirable applications. CPCs can also find utilization in other tissue engineering areas such as macroporous scaffold development, strengthening relationships between other processed structures, and in supplying growth factors and cells [137].

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Cellular response to bioactive glasses and glass–ceramics

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18.1 Introduction

Immunological responses start to happen in the body system as the biomaterial is implanted in the body. These responses, commonly interrupt the in vivo functionality and durability of the implanted biomaterials [1,2]. By implantation of biomaterials, the adsorbed proteins, along with macrophages and dendritic cells (DCs) induce interactions between cells and biomaterials [3]. Rapid adsorption of proteins on the surface of the implanted biomaterials activates foreign body responses (FBRs) mechanisms. Functionality of the adsorbed proteins is greatly dependent on the physicochemical and biological properties of the implanted biomaterial surface and proteins' properties [4,5]. Therefore the immunological system responses to the surface of a biomaterial determine the applicability of the implanted material. When exposed to the biological conditions, a progressive response occurs at the surface of implanted bioactive material through precipitation of a biologically active layer [6]. This porous layer, known as hydroxycarbonate apatite (HCA), surrounds the bioactive implanted materials and starts to make connections with the neighboring tissues [6]. On the basis of mechanism of interaction in biological conditions, ceramic biomaterials are divided into two different main groups. In the first group, bioactivity is the inherent feature of material and originates from its chemical properties; in the second group, bioactivity is stimulated through surface modification techniques as well as the addition of some activating agents to the structure of the intended ceramic [7,8]. Among the biologically active ceramics, bioactive glasses and glass–ceramics have been

greatly attended from the past few decades, owing to their ability to make connections with the neighboring hard and soft tissues [9–11]. These materials could be widely used in the field of tissue engineering.

Glass–ceramics are polycrystalline materials that contain crystal phases, homogeneously distributed in the remained glass matrix [12]. These materials are commonly fabricated by controlled crystallization of a parent glass. Whereas the parent glass is used as the powder (e.g., sol–gel derived glasses), concurrent crystallization and sintering take place at the same heat treatment procedure [7,8]. The crystallinity of glass–ceramics could be controlled by changing the chemical composition of the parent glass and heat treatment parameters including heating rate, soaking temperature, and soaking time. The common volume crystallinity of most glass–ceramics falls in the range of 30%–70%. Accordingly, a vast range of bioactivity with totally different biological and physicochemical features could be easily obtained from the glass–ceramics with different crystallinity [13,14].

This chapter focuses on the applications of bioactive glasses and glass–ceramics in the field of tissue engineering, discussing the FBRs mechanisms. Moreover, considerable examples of interactions between cells and biomaterials will be reviewed. In this regard, the main concern of the present chapter is to explain the biocompatibility responses of various bioactive glasses and glass–ceramics having different chemical compositions and physicochemical features. Finally, some promising strategies to promote protein adsorption and cellular functionality of the implanted materials will be introduced.

18.2 Biological responses to biomaterials

When a biomaterial is implanted inside the body, the process of tissue/material interface formation appears [15]. There are nonspecific blood and tissue fluid proteins absorbing on the biomaterial's surface. They act as the key players in interactions between cells and the biomaterials surface [16,17]. The biomaterials' physicochemical properties have direct effects on the FBRs [3,17]. When a biomaterial is implanted in a body, it can cause injury, which leads to accumulation of proteins, macrophages, and DCs. The process of protein accumulation can direct the wound healing reactions as well as acute and chronic inflammatory mechanism [18]. The wound site cleaning and matrix formation (i.e., acute inflammation phase) could vary from hours to days. The adsorption of proteins, macrophages type 1, and neutrophils control the inflammation phase. Body vessels expand during the process of acute inflammation phase, which leads to streaming extra blood to the injured area [1,3,18]. Then, numerous blood and tissue proteins including albumin, fibronectin (FN), fibrinogen, vitronectin, and complement C3 are released. When monocytes are present in the wound healing area, they can differentiate into macrophages type 1 (M1) and type 2 (M2) [3,5,18,19]. M1 directs acute inflammatory stage by producing proinflammatory factors, while M2 directs chronic inflammatory phase by producing anti-inflammatory factors. It has been clear that continual subsistence of biomaterial or tissue interfaces result in M2 activities and chronic inflammatory responses [19,20]. Besides, in terms of histology, the surrounded tissue in chronic inflammation phase is less than acute inflammation. This swelled tissue originates from the release of lymphocytes, monocytes and M2, and formation of connective tissue and blood vessels [15,21].

In point of the injury's degree at the implanted area, the period of acute phase lasts about less than 1 week, while the period of the chronic phase lasts about 2 weeks. Then, an extracellular matrix (ECM) is replaced by the granulation tissue, which can be considered as a physical scaffold and plays an important role in the biological responses [19]. The fibroblasts infiltration and neovascularization are identified during the inflammatory responses and the colony of macrophages. The formation of fibrous capsule occurs before tissue granulation. This fibrous capsule is then separated from the surface of biomaterials via the cell constituents [18]. The total period of all inflammatory reactions at the biomaterial's surface is always less than 3 weeks. The physicochemical characteristics of biomaterials such as bioactive glasses and glass–ceramics have a direct effect on the protein adsorption, which leads to their biological responses, which will be discussed in the next sections.

18.3 Bioactive glasses and glass–ceramics: structure and their physicochemical properties

A bioactive material has the capability of HCA formation on the surface, when it is exposed to the biological conditions. Formation of HCA layer on the surface of a bioactive material originates from continuous interactions between preferential surface sites and cells [22]. HCA formation ability of a material after immersion in a simulated body fluid (SBF) *in vitro*, could be a rough estimation about its bioactivity [22]. It is worthy to note that on the basis of *in vitro* bioactivity assessment, giving an accurate estimation about functionality of the examined material in the biological conditions is not reliable [23,24]. Bioactive glasses and glass–ceramics, having considerable apatite formation ability, have been greatly intended by scientists and surgeons in both *in vitro* and *in vivo* examinations [6,24,25]. Similar to other oxide glasses, bioactive glasses are composed of three different constituents: glass formers [including silica (SiO_2), boric acid (B_2O_3), and phosphoric oxide (P_2O_5)], network modifiers (including alkaline and alkaline earth oxides) and intermediate oxides (including transition metal oxide) [26]. In the structure of bioactive glass, tetrahedral units are connected to each other by bridging oxygens. In each tetrahedron, glass former cation is located at the center and surrounded by oxygens at the corners. Through linking to the oxygens, modifier cations turn the bridging oxygen to the nonbridging ones. It is worth mentioning that the presence of considerable amounts of modifier oxide in the glass composition, considerably weakens the glass structural bonds and declines mechanical properties [27]. Fig. 18.1 schematically shows the glass structure consisting of the as-mentioned different constituents. According to this figure, silicon and phosphorous as the network formers occupy the center of tetrahedral units; oxygen locates at the corner of each tetrahedron. Sodium, calcium, and other additive ions are connected with nonbridging oxygen.

By exposure to the crystallization heat treatment, bioactive glasses transform to the glass–ceramic materials with improved mechanical properties and modified bioactivity. In the following sections, the physicochemical properties of main types of bioactive glasses and glass–ceramics with different biological responses will be discussed.

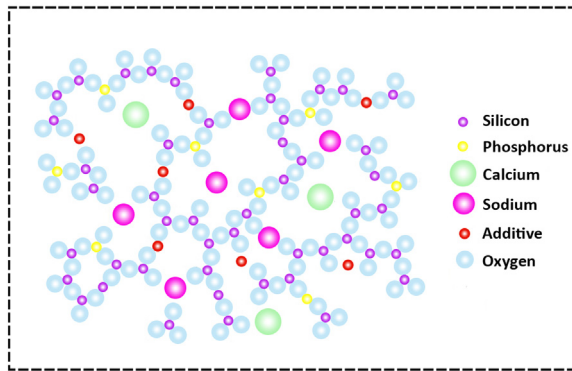


FIGURE 18.1 Schematic structure of a bioactive glass: tetrahedral units (SiO_4) are connected to each other by bridging oxygen. In each tetrahedron, glass former cations (silicon and phosphorous) are located at the center, being surrounded by oxygen at the corners. Through linking to the neighboring oxygen, modifier cations (sodium, calcium, and other additive ions) turn the bridging oxygen to the nonbridging ones. Source: *Authors*.

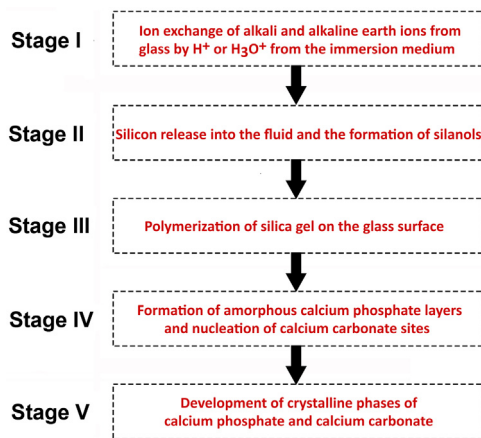


FIGURE 18.2 An overview of sequential reactions occurring on the surface of bioactive glasses in contact with body fluids, followed by formation of crystalline phases including calcium phosphate (usually as the carbonated hydroxyapatite) and calcium carbonate (usually as the calcite) on the surface of bioactive glasses. Source: *Authors*.

18.3.1 Silicate-based glasses

In the late 1960s, the first successfully experienced silicate-based bioactive glass was introduced by Hench et al. for the first time. This glass, known as the 45S5 bioactive glass with the chemical composition of 45SiO_2 , $24.5\text{Na}_2\text{O}$, 24.5CaO , and $6\text{P}_2\text{O}_5$ (in wt.%), is capable to chemically bond to the surrounding tissues [28,29]. Appropriate bioactivity of the 45S5 glass is mainly originated from the restrictedly designed chemical composition including low SiO_2 content, high Na_2O and CaO contents, along with controlled ratio of $\text{CaO}/\text{P}_2\text{O}_5$ [24]. During exposure to the biological conditions, formation of a HCA layer on the surface of the bioactive glass proves to be the main parameter which stimulates connections between the glass surfaces and surrounding hard and soft tissues [7,8,30]. As suggested by Hench et al. [30], the formation of HCA layer is in turn caused by a series of consequent reactions on the surfaced of implanted bioactive glass. Fig. 18.2 briefly shows the different stages of apatite formation on the surface of bioactive glasses. In stage I, alkali and alkaline earth ions (Na^+ and Ca^{2+}) diffuse from the glass structure into the surrounding medium through an ion exchange process. As a result, H^+ or H_3O^+ enters into the

glass structure from the medium. In this state, an increase of the pH value leads to gradual dissociation of Si–O–Si bonds. By the time, release of silicon ions to the medium and formation of silanols (Si–OH groups) on the glass surface occurs in stage II. In stage III, a silica gel layer is formed on the glass surface caused by silanol groups' condensation. In stage IV, simultaneous diffusion of Ca^{2+} and PO_4^{3-} ions from both the glass structure and the surrounding medium provides an amorphous layer of calcium phosphate and calcium carbonate over the silica gel layer. In stage V, crystallization of the as-mentioned amorphous layer takes place and calcium phosphate (usually as the carbonated hydroxyapatite) and calcium carbonate (usually as the calcite) start to crystallize on the glass surface [31].

Formation of an apatite layer on the surface of bioactive glasses activates the growth factors and consequent cells responses [32]. Gradual dissolution of bioactive glasses is accompanied by the formation of ECM that forms both crystalline phase and collagen on the glass surface [33]. In point of bioactivity, the 45S5 glass is still unsurpassed in comparison with other bioactive glasses and has been widely targeted by several studies to highlight its interaction with molecules and cells in biological conditions [34–36]. Contrary of this outstanding priority, utilizing 45S5 glass in biomedical applications encounters some serious difficulties. The main drawback of the 45S5 glass is attributed to the restricted temperature interval between the glass transition temperature (T_g) and onset of crystallization temperature (T_c). This narrow temperature interval causes the interference between densification and crystallization steps. When the densification through viscous flow mechanism is interrupted by crystallization, the glass could not be densified further. As a result, mechanical properties will fail and not be satisfying [37]. Moreover, due to the difference between chemical composition of implanted 45S5 glass and body tissues, the inconsistency between degradation of bioactive glasses and surrounding tissues is unavoidable [38]. On the other hand, the implanted bioactive glasses do not completely transform to an HCA layer and leave some unreacted SiO_2 in most cases, which should be considered in terms of cytotoxicity. Additionally, the change in the concentration of some ions released from the glass structure may alter the pH value and also need more consideration in point of cytotoxicity [38–40]. Therefore in the last two decades, a progressive focus was put on the development of a new glassy material having simultaneously considerable bioactivity (comparable to 45S5 Bioglass) and mechanical properties (comparable to apatite/wollastonite Cerabone).

In this regard, Zanotto et al. modified the chemical composition of 45S5 glass into 23.75 Na_2O , 23.75 CaO , 48.5 SiO_2 , and 4 P_2O_5 (in wt.%). Through a controlled double step heat treatment, they could transform the initial glass into the spectacular glass–ceramic product with outstanding properties. This new class of bioactive glass–ceramics, known as Biosilicate, contains one (sodium calcium silicate: $\text{Na}_2\text{CaSi}_2\text{O}_6$) or two (both sodium calcium silicate and sodium calcium phosphate: NaCaPO_4) crystalline phases depending on the applied heat treatment procedure [41]. Biosilicate exhibits a wide range of properties essentially required for tissue engineering applications including remarkable bioactivity, osteoconductivity, osteoinductivity, noncytotoxicity, nongenotoxicity, and antibacterial effects along with excellent machinability. In the form of monolithic glass–ceramic, Biosilicate is tough and strong enough. It can also be utilized as the powder to synthesize scaffolds through various techniques [41]. Crovace et al. fabricated glass–ceramic scaffolds from Biosilicate composition through foam replication method. After sinter-crystallization

heat treatment, the obtained glass–ceramic scaffolds contained total porosity of about 92% and pore size in the range of 400–900 μm [42] (see Fig. 18.3A).

After in vitro bioactivity assessment for different time periods, HCA layer created on the surface of the scaffold structure, as shown in Fig. 18.3b and c. In another research, Moura et al. examined the in vitro osteogenesis capability of Biosilicate glass–ceramic using rat calvaria bone cells for up to 17 days. Based on the obtained results, Biosilicate indicated considerable increase of both calcified tissue areas and calcified matrix in comparison with 45S5 glass and Biosilicate parent glass (see Fig. 18.3d) [43]. It was also found that the osteoblastic MC-3T3 could successfully grow on the laser irradiated Biosilicate glass–ceramic scaffolds [44] (see Fig. 18.3e). However, laser irradiation decreased cell growth compared to the nonirradiated specimens.

18.3.2 Borate-based glasses

Borate-based glasses are reckoned as the specific type of bioactive glasses having glass network totally different from that of silicate and phosphate-based bioactive glasses [45,46]. The network of borate-based glasses consists of trigonal BO_3 units, connected to each other by oxygen ions shared between two neighboring units. By entrance of alkali and alkali ions into the network structure, an abnormal structural change occurs in the structure of borate-based glasses. In this status, unlike silicate and phosphate-based glasses, the number of nonbridging oxygen will be decreased via turning trigonal BO_3 units into tetrahedral BO_4 ones [26]. It is possible to control the degradation rate of borate glasses by changing of the chemical composition. As an instance, degradation rate of 45S5 or 13-93 glasses is highly dependent on the partial replacement of SiO_2 by B_2O_3 in the chemical

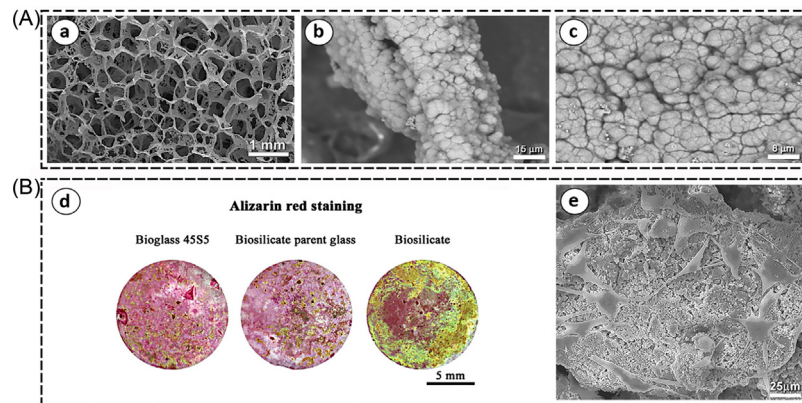


FIGURE 18.3 (A) SEM micrographs of the Biosilicate glass–ceramic scaffold (a) before and after SBF immersion for (b) 3 day and (c) 7 days. (B) Osteogenesis assessment: (d) Macroscopic images of osteogenic cultures grown on the 45S5 Bioglass, Biosilicate parent glass and Biosilicate glass–ceramic, stained with Alizarin red at day 17. (Bone-like matrix formation is shown by yellowish areas in a reddish background.) (e) SEM micrograph of the osteoblastic cells grown on a Biosilicate glass–ceramic scaffold. SBF, Simulated body fluid. Source: Reproduced with the permission from Crovace MC, et al. Biosilicate®—a multipurpose, highly bioactive glass–ceramic. *In vitro, in vivo and clinical trials*. *J Non-Cryst Solids* 2016;432:90–110. ©2019 Elsevier.

composition of the starting glasses. This priority as well as the possibility to vastly design the chemical composition of bioactive borate-based glasses make them wise choices for biomedical applications [26,40]. Interestingly, more bioactivity of borate-based glasses has been noticed in comparison with that of silicate-based 45S5 [47]. Some research has focused on the suitability of borate bioactive glasses in point of in vitro cell growth and in vivo tissue infiltration concerning treatment of bone defects. Deliormanlı et al. [48] have examined the porous borate-based bioactive glass scaffolds containing different amounts of cerium oxide (1, 3, and 5 wt.%), gallium oxide (1 and 5 wt.%), and vanadium oxide (0.15, 1, and 3 wt.%), in terms of soft tissue ingrowth and angiogenesis. In this regard, they synthesized different 13-93B3-based glasses through the classic melt quenching technique. Different borate-based glass scaffolds were fabricated via foam replication method. For each series of glass composition, the corresponded fabricated scaffolds were subcutaneously implanted in four sites into the connective tissues of male rats. After 4 weeks of implantation, histology analysis was used to determine tissue ingrowth and blood vessels creation in the implanted scaffolds. On the basis of histological observation, in cerium containing glasses as well as the control group sample (bare 13-93B3), considerable amounts of blood vessels were detected. The quantified results showed that by increase of cerium content from 1% to 5%, the blood vessel area percentage increased from 13.90% to 16.46% (see Fig. 18.4).

In another research, Cui et al. [49] have evaluated the bone formation capability of an injectable bioactive cement composed of borate glass and chitosan bonding solution in a rabbit femoral condyle model, in comparison with a commercial calcium sulfate cement as the control sample. Interestingly, the applied glass cement showed a superior bone formation ability after 12 weeks of injection. They surveyed the morphological aspects of the reconstructed femoral tissues by using micro-CT. The formation of newly formed blood

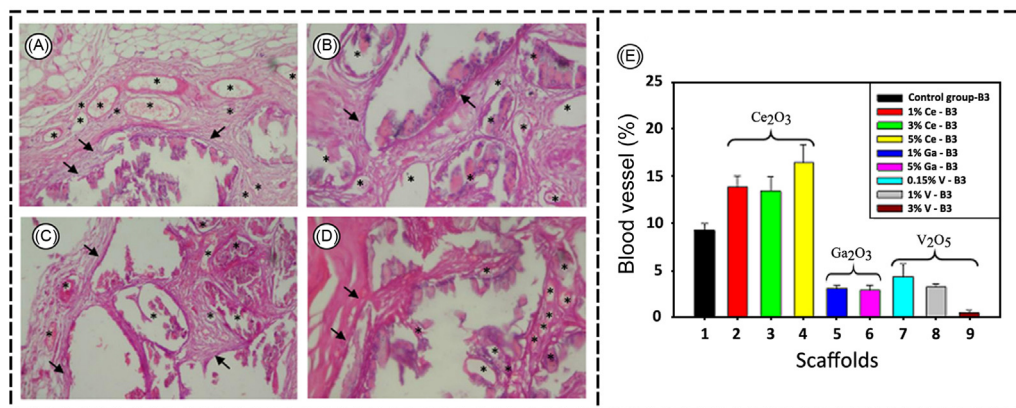


FIGURE 18.4 Optical microscope images of the H&E stained samples: (A) control sample (bare 13-93B3), (B) 1% Ce-13-93B3, (C) 3% Ce-13-93B3, and (D) 5% Ce-13-93B3, arrows: borderline of the scaffold, *blood vessels. Cytoplasm connective tissue and extracellular matrix are shown as purple or pink, and red blood cells are shown by bright red dots. (E) The total blood vessel area in the borate-based bioactive glass scaffolds. Source: Reproduced with the permission from Deliormanlı AM, et al. *In vivo* evaluation of cerium, gallium and vanadium-doped borate-based bioactive glass scaffolds using rat subcutaneous implantation model. *Ceram Int* 2016;42(10):11574–83. ©2019 Elsevier.

vessels after post implantation was examined by histology analysis (see Fig. 18.5). In the micro-CT images taken from tissues implanted with bioactive glass and calcium sulfate cements, three different areas were detectable: host bone, the remained bone cement, and the freshly formed bone. According to the micro-CT imaging, the bioactive glass cement had a lower degradation rate at 4 and 8 weeks after implantation. But, at 12 weeks, the area of newly formed bone was considerably larger rather than the calcium sulfate cement control sample. This finding was also confirmed by histology analysis [49] (see Fig. 18.5).

On the other hand, weak cellular response to bioactive borate-based glasses has been also reported. As an example, Lopes et al. [50] examined the cytotoxicity of MG-63 and

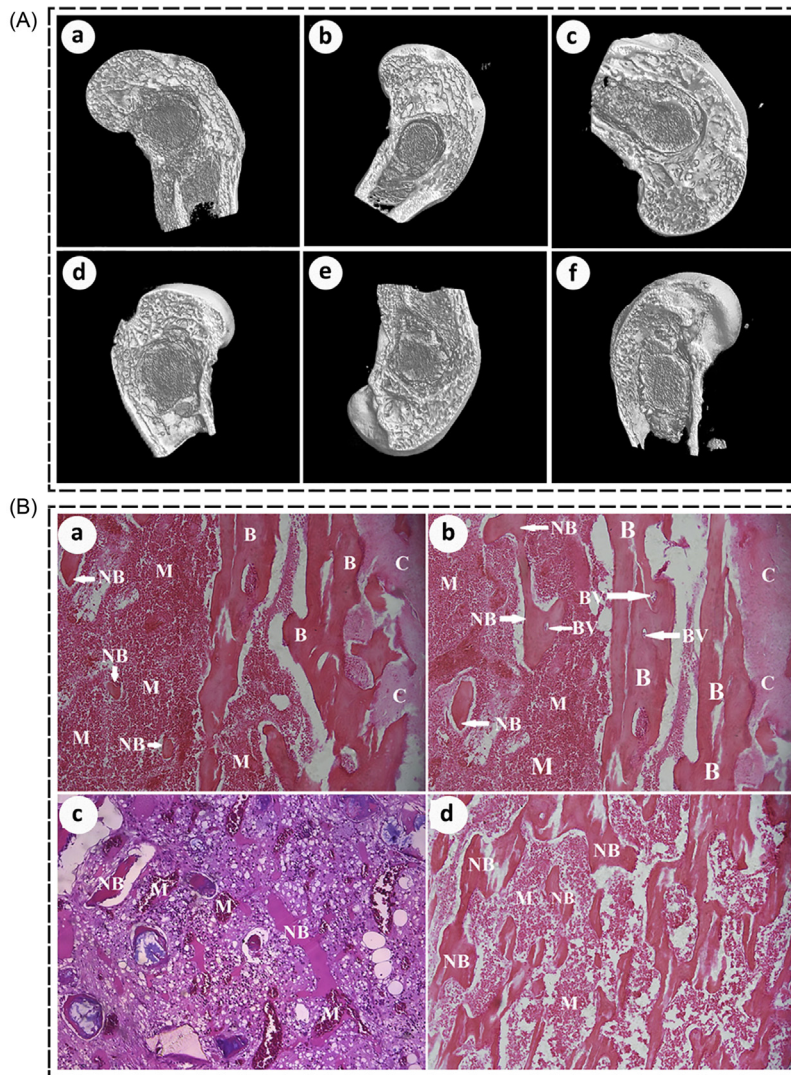


FIGURE 18.5 (A) Micro-CT images taken from rabbit femoral condyles implanted with bioactive borate glass cement for (a) 4 weeks, (b) 8 weeks, and (c) 12 weeks compared with calcium sulfate cement for (d) 4 weeks, (e) 8 weeks, and (f) 12 weeks. (B) Optical microscope images of the H&E stained sections of rabbit femoral condyles implanted with (a, b, and d) borate bioactive glass cement and (c) calcium sulfate cement at 12 weeks. M, cement; B, host bone; BV, blood vessel; NB, new bone; C, cartilage. [Magnification: (a, b, and c) $\times 100$ and (d) $\times 400$]. Source: Reproduced with the permission from Cui X, et al., *Evaluation of an injectable bioactive borate glass cement to heal bone defects in a rabbit femoral condyle model*. *Mater Sci Eng C* 2017;73:585–95. ©2019 Elsevier.

hMSCs responses to different silicate and borate-based bioactive glasses. Although silicate glasses showed acceptable cellular responses, borate specimens had an adverse trend, probably due to the higher degradation rate and increased amounts of B and Mg ions in the surrounding medium. It should be noticed that application of borate-based glasses is still doubtful, in point of cytotoxicity.

18.3.3 Phosphate-based glasses

Considering significant solubility, biocompatibility, biodegradability, as well as having well-matched chemical composition with the mineral portion of bone tissues, phosphate-based glasses have been progressively attended for tissue engineering and regenerative medicine applications [6,51]. Similar to the silicate-based glasses, the structure of phosphate-based glasses consists of tetrahedrons linking together via oxygen ions.

In this type of bioactive glasses, phosphorous is the network former cation, while calcium and sodium ions take part in the glass structure as the modifiers. Due to the more dissociated glass network rather than silicate glasses, phosphate-based glasses suffer from lower chemical resistance which is not appreciated for biomedical applications [52]. However, it is possible to adjust the chemical stability of bioactive phosphate-based glasses through modification of glass composition representing these glasses as resorbable biomaterials [24]. As an instance, by decrease the CaO content in the glass composition, increase of solubility and biocompatibility of phosphate bioactive glasses accompanied by lowered dissolution rate and increased proliferation of osteoblast cells have been demonstrated [53,54]. On the other hand, the presence of different amounts of Na₂O in the composition of bioactive phosphate-based glasses led to decreased cell adhesion and osteoblast cells' proliferation [55].

18.4 Innovative strategies for selective contribution of bioactive glasses

Over the past decade, plenty of research has focused on chemical modification of bioactive glasses, in order to promote the cellular and molecular interactions. Composition modification with different ions (ion doping), silanization, along with biological functionalization of bioactive glasses are the main successful tactics that will be further discussed in the upcoming sections.

18.4.1 Cellular and molecular behavior of bioactive glasses in response to different doped ions

Modification of bioactive glasses through ion doping makes it possible to induce their specific cellular and molecular responses. Ion doping could significantly alter the network connectivity, solubility, and biological properties of the targeted bioactive glasses. In addition, some researchers have reported that incorporating some special ions into the network of bioactive glasses might reinforce glass structural bonds and improve mechanical

properties, consequently. Undoubtedly, these modifications affect the biological properties of bioactive glasses.

18.4.1.1 Fluoride-containing bioactive glasses

As an advantageous doping agent, fluoride (F) ions have been appreciated owing to partially contribution to the crystal structure of HA and conversion of HA phase of calcified tissue into fluorapatite (FA) [56–58]. Compared to HA, FA presents higher physicochemical stability which makes it more resistant against acidic solutions [59]. Incorporation of fluoride ions into the structure of bioactive glasses could be especially useful for treatment of dental caries as one of the most common bone tissue defects. Dental caries originate from demineralization and destruction caused by an acidogenic bacteria [60]. In this regard, fluoride ions have antimicrobial effects and make apatitic structure more resistant against acidic demineralization [61]. However, the cytotoxicity of fluoride ions should be considered [62]. The cytotoxicity of fluoride is greatly determined by its concentration. Low concentrations of fluoride ions improve the osteoblast cells growth, whereas high concentrations impede the cells' growth. A recent study shows the effects of fluoride addition on biological and mechanical properties of bioactive glass–ceramics belonging to the Na_2O – CaO – SiO_2 – P_2O_5 system [63]. The authors prepared different series of glass–ceramic specimens doped with NH_4HF_2 (G- NH_4HF_2) and CaF_2 (G- CaF_2). In comparison with NH_4HF_2 doped glass–ceramic, CaF_2 doped sample showed better mechanical properties and inferior biocompatibility. Interestingly, both glass–ceramics had the thermal expansion coefficients close to that of Ti6Al4V. Furthermore, subsequent in vitro and in vivo bioactivity assessments confirmed the biocompatibility and appropriate osseointegration of the studied bioactive glasses.

In another research, Gentleman et al. fabricated a series of bioactive SiO_2 – P_2O_5 – CaO – Na_2O glasses contained different amounts of CaF_2 (1, 4.75, 9.28, 13.62, and 17.76, in mol.%), with constant network connectivity (constant ratio of glass former to glass modifier) [64]. As can be seen in Fig. 18.6, Saos-2 cells attached to the surface of bioactive glasses were morphologically normal after 7, 14, and 28 days in culture. Fluorescence staining of live and dead cells on the surface of bioactive glass disks confirmed considerable differences in cell attachment, morphology, and proliferation. In control group samples (without CaF_2), limited cell attachment and proliferation were observed. However, the addition of 1 mol.% CaF_2 , resulted in the increased cell proliferation, especially at later time points. After 28 days in culture, cellular aggregates were formed on the surface of glasses contained more than 9.28 mol.% CaF_2 [64] (see Fig. 18.6).

18.4.1.2 Magnesium containing bioactive glasses

Among therapeutic metallic ions, magnesium (Mg) is crucially important and could enhance both stability of nucleic acids and the ions metabolisms in body [26,65]. By increase of osteoblasts' activity, it could also reinforce bone formation [66]. Considering these priorities, numerous researchers have focused on the incorporation of magnesium ions into the bioactive glass network. However, there are serious contradictions about structural role of magnesium ions in the glass network and consequent effects of these ions on physicochemical and biological properties of bioactive glasses.

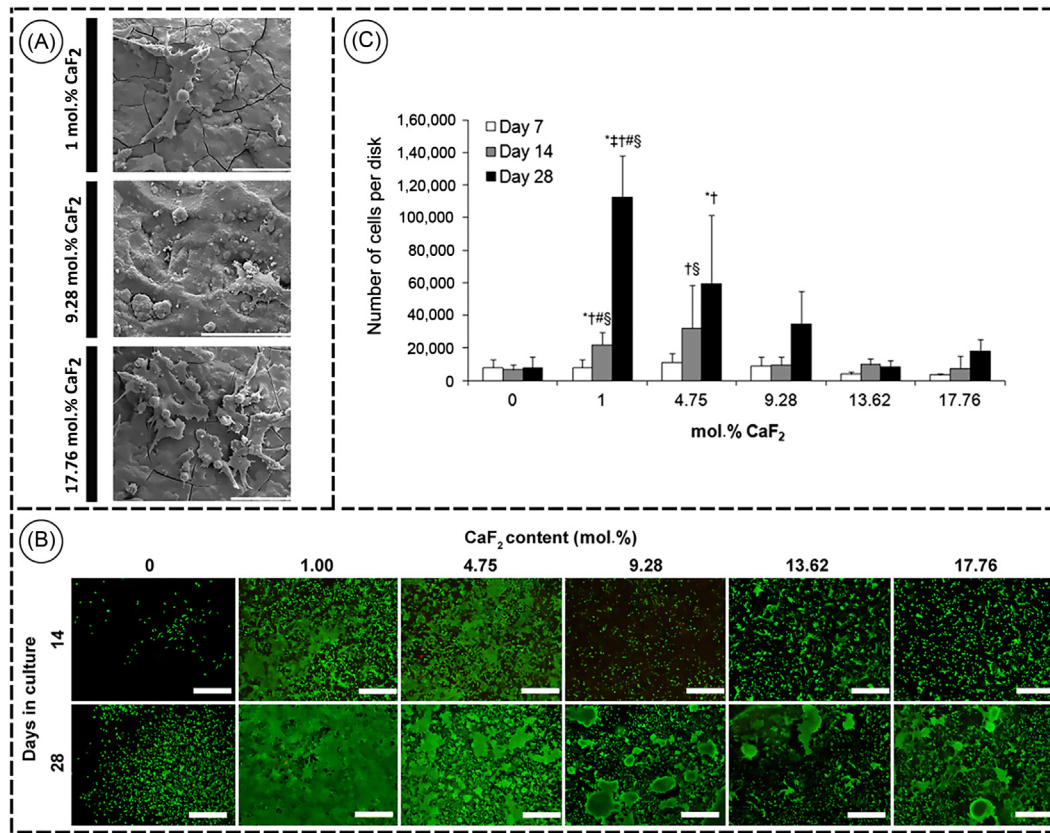


FIGURE 18.6 (A) SEM micrographs of cells adherent to the surface of bioactive glasses containing 1.00, 9.28, and 17.76 mol.% CaF₂ after 14 days in culture; (B) cell viability staining of Saos-2 cells adherent to the surface of bioactive glasses after 14 and 28 days in culture (live cells appear green whilst dead cells are stained red. Scale bar = 500 μ m); (C) total number of Saos-2 adherent to BG disks after 7, 14, and 28 days in culture. *Significantly more cells in the indicated group compared to the 0 mol.% CaF₂ group at the same time point. †Significantly more cells than the 9.28 mol.% CaF₂ group at the same time point. ‡Significantly more cells than 13.62 mol.% CaF₂ group at the same time point. §Significantly more cells than 17.76 mol.% CaF₂ group at the same time point. Source: Reproduced with the permission from Gentleman E, et al., Surface properties and ion release from fluoride-containing bioactive glasses promote osteoblast differentiation and mineralization in vitro. *Acta Biomater* 2013;9(3):5771–9. ©2019 Elsevier.

From different research, some of them suggested magnesium ion as the modifier, and other ones introduced it as an intermediate agent, that could form MgO₄ tetrahedrons in the glass network [67–69]. Furthermore, the positive effect of magnesium ions on enhancement of physical features of bioactive glasses including surface area and porosity has been reported [70]. However, another investigation showed contradictory results indicating imperceptible effects of magnesium ions on physical properties [71] along with reduced degradation rate and subsequently postponed apatite formation on the surface of bioactive glasses [40,71,72].

Regardless of the lack of enough supported data to solve the mentioned contradictions, some researchers believe that magnesium ions could increase the adhesion of osteoblastic cells and improve proliferation and other biological responses to bioactive glasses [73]. In this regard, Al-Noaman et al. [74] fabricated a series of bioactive glasses containing different concentrations of magnesium ions and examined the obtained glasses in terms of thermal properties, structural features, and bioactivity. Based on the obtained results in this research, by increase of magnesium content, thermal expansion coefficient, glass transition, and crystallization peak temperatures reduced. Interestingly, magnesium containing glasses did not hinder the apatite formation. Further biological examinations confirmed appropriate responses of fibroblast cell to magnesium-doped bioactive glasses. However, osteoblast cells' responses to them were not satisfying [74,75].

18.4.1.3 Strontium containing bioactive glasses

Strontium (Sr), as one of the main constituents of the bone tissues has a direct effect on metabolism of the bone [25,76,77]. The needed amounts of strontium ions for osteoporosis treatment are usually provided from strontium ranelate and strontium chloride as the commercial agents. In order to improve bone reconstruction, numerous research has focused on the substitution of calcium ions by strontium ions in the structure of bioactive glasses [25,76,77]. These chemically modified bioactive glasses could be applied for treatment of vertebral compression fractures [78]. Furthermore, strontium containing bioactive glasses could keep the bone tissues away from bacteria and microbial infections [25,76]. The presence of strontium ions in the network of bioactive glasses induces antimicrobial activity against subgingival bacteria, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. This effectiveness is in direct relationship with the concentration of Sr in the glasses [79]. In point of biocompatibility of strontium-doped bioactive glasses, the concentration of doped ions has a crucially vital role. Low concentrations of strontium ions enhance the bone formation, whilst, high concentrations may cause abnormality of bone [80]. It has been confirmed that bioactive glasses containing less than 5 mol.% SrO indicate greater cell proliferation and alkaline phosphatase (ALP) activity compared to strontium-free glasses or glasses having higher concentration of strontium ions [81]. According to some research, by incorporation of strontium ions into the network of bioactive glasses and replacement of calcium ions by these entering ions, the network compaction would be increased owing the larger ionic radius of strontium ion rather than calcium ion. As a result, the glass structural density and oxygen density would be increased and decreased, respectively [82].

18.4.1.4 Silver-containing bioactive glasses

Silver (Ag) ion is well known as one of the most effective therapeutic ions indicating considerable antibacterial properties [58,83–85]. Several researches have pointed out that silver-containing bioactive glasses could restrict bacteria growth (including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) when contacted with the biological surrounding [86–88]. Additionally, silver-doped bioactive glasses present antiinflammatory effects. Considering both antibacterial and antiinflammatory properties of silver-doped bioactive glasses, this class of bioactive glasses seems as a promising choice for tissue reconstruction and especially wound healing uses. It has been confirmed that with respect

to the base silver-free bioactive glasses, silver-doped ones showed superior antibacterial and biological properties [89]. Goh et al. [90] have examined the antibacterial properties of silver and copper-containing bioactive glasses. In contact with the biological surrounding, silver ions showed a considerably higher rate of release rather than copper ions. However, copper ions had an extended ion release behavior. Therefore silver and copper-containing bioactive glasses were proposed for rapid and long-term antibacterial activity, accordingly. The effectiveness of small amounts of silver ions on antibacterial properties of the corresponded bioactive glasses without any toxicity has been also reported [83,91,92].

In another study, Chamorro et al. [93] have utilized silver-doped bioactive glass–ceramics (Ag-BG) microparticles showing antibacterial properties combined with various antibiotics. They have developed this complicated system to overcome methicillin-resistant *S. aureus* (MRSA). In this multifunctional system, those antibiotics were applied that previously showed resistance against (MRSA), including oxacillin or fosfomycin, and vancomycin. The results of electron microscopy observations and bacteria viability assay have demonstrated that sol–gel derived Ag-BG microparticles combined with antibiotics decreased the viability of MRSA (see Fig. 18.7).

18.4.1.5 Copper-containing bioactive glasses

Copper (Cu) is another metallic therapeutic ion indicating antibacterial properties [90,94]. Copper could significantly eradicate *E. coli*, MRSA, and *Clostridium difficile* [95,96]. Some previous researches have confirmed that the presence of copper ions in the composition of bioactive glasses could stimulate the cell responses, thanks to the antibacterial

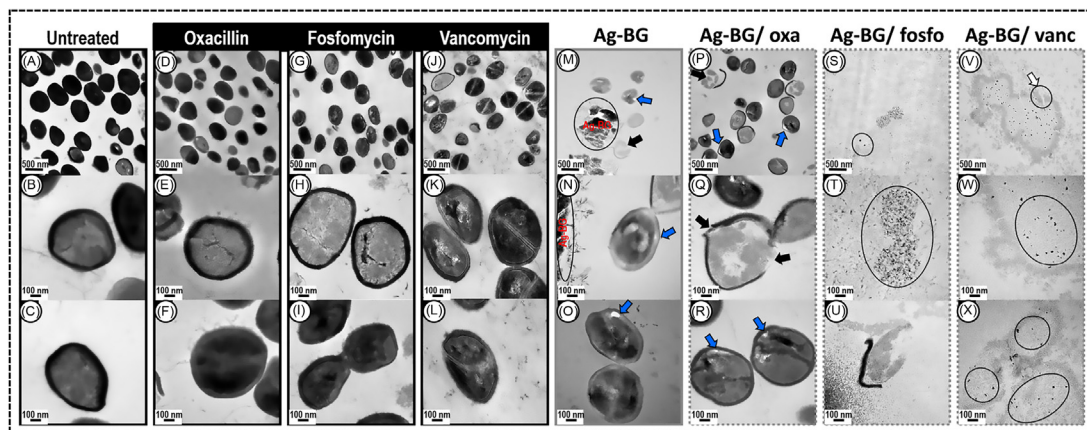


FIGURE 18.7 TEM images of bacteria untreated (A–C), after being exposed for 24 hours to oxacillin alone (D–F), fosfomycin alone (G–I), vancomycin alone (J–L), Ag-BG microparticles alone (M–O), or to the combinations: Ag-BG/oxa (P–R), Ag-BG/fosfo (S–U), and Ag-BG/vanc (V–X). Ag-BG microsized and nanosized particles were marked with black lines. Black arrows point out damaged cells. Blue arrows indicate the void formation between the cell envelope and cytoplasm, and white arrows mark a nanotunnel/channel. Source: *Reproduced with the permission from Pajares-Chamorro N, et al. Resurrection of antibiotics that methicillin-resistant Staphylococcus aureus resists by silver-doped bioactive glass-ceramic microparticles. Acta Biomater 2019;96:537–46. ©2019 Elsevier.*

effects of copper. With bioactivity, it should be considered that the apatite formation capability of copper-containing bioactive glasses is critically dependent to the applied dose of copper ions. Increased concentration of copper could suppress apatite formation [97]. It has been shown that Cu ions commonly contribute into the network of bioactive glasses as a modifier agent, having less structural effects compared to magnesium and zinc ions [98]. Similar to other therapeutic ions, the efficacy of copper ions is in direct relationship with the chemical composition of the host bioactive glass. Apart from antibacterial effects, copper ions can promote osteogenesis and angiogenesis, at the same time. Wang et al. [94] have synthesized copper-containing (0–3.0 wt.% CuO) borate-based bioactive glasses. Utilizing these glasses and by the foam replication technique, they fabricated bioactive glass scaffolds. By immersion of the prepared scaffolds in the SBF solution, the release of copper ions to the solution initiated, while the rate of copper release was totally governed by the content of copper ions. When exposed to the in vitro cytotoxic tests on hBMSCs (human body mesenchymal stem cells), the applied concentrations of Cu ions did not show any cytotoxic effects and even enhanced the ALP activity. Moreover, the examinations of the implanted scaffolds in a rat model confirmed increased angiogenesis ability of 3 wt.% CuO containing bioactive glass, in comparison with the copper-free base glass.

In another research, Ryan et al. [99] have introduced a new one-step treatment for osteomyelitis. They have loaded copper-free (BG) and copper-containing (CuBG) bioactive glasses into the collagen-based scaffolds and examined their antibacterial behavior, osteogenesis, and angiogenesis activity, both in vitro and in vivo. In this regard, they firstly synthesized the collagen scaffolds via freeze drying method. They have also prepared two different series of copper-free and copper-containing bioactive glasses through sol–gel process. Chemical composition of the mentioned glasses have been respectively as $60\text{SiO}_2\text{--}36\text{CaO--}4\text{P}_2\text{O}_5$ and $60\text{SiO}_2\text{--}34\text{CaO--}2\text{CuO--}4\text{P}_2\text{O}_5$ (in mol.%). Afterwards, they loaded different contents of both series of glasses into the collagen scaffolds. It has been reported that 300% CuBG was the maximum content of bioactive glass that could be loaded into the collagen scaffold. Collagen-CuBG scaffolds showed antibacterial activity against *S. aureus* along with enhanced osteogenesis and angiogenesis, in vitro. Additionally, when examined in chick ex vivo embryo model, they have also shown considerable osteogenesis and angiogenesis activity, in consistently with the in vitro assessment. In order to monitor angiogenesis capability of the studied scaffolds, the chorioallantois membrane (CAM) was analyzed in terms of quantified blood vessel density. Fig. 18.8 represents the results of angiogenesis assessment, taken from the scaffolds and CAM of all chick embryos. In this assessment alone, collagen scaffold and VEGF (vascular endothelial growth factor)-loaded scaffold were used as the control group. Among the examined scaffolds, 100% CuBG scaffolds have shown considerably increased angiogenesis activity. Surprisingly, these series of scaffolds have been vascularized even more than VEGF-loaded control.

It should be taken into account that the effects of copper ions on vascularization originates from the ability to produce VEGF which is absolutely essential in reconstruction of the targeted tissues. Aside from antibacterial potential of copper ions, their cytotoxicity effects on healthy cells should be considered. Therefore the applied dosage of Cu ions is critically important.

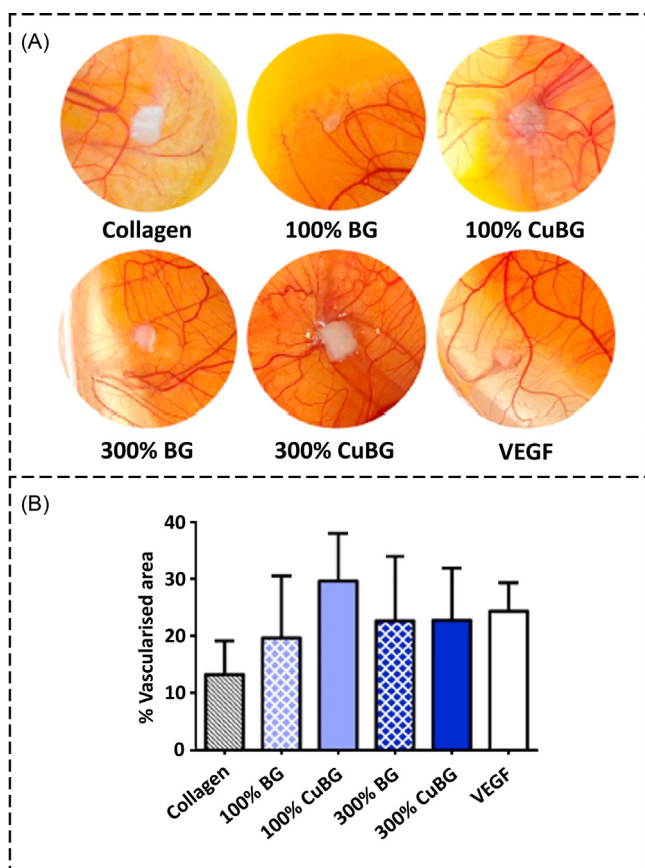


FIGURE 18.8 Effect of bioactive glass scaffolds on angiogenesis in a chick embryo ex vivo model: (A) Representative images of the angiogenic response to bioactive glass scaffolds on the surrounding CAM membrane with collagen only and VEGF scaffolds as controls. (B) Quantified percentage vascularized area in a circular area around the scaffold treatments. Data presented as mean $\pm$ SD, $n = 3$, P -values are calculated using an unpaired two-tailed t -test with 95% CI. All statistical significance shown in comparison to collagen control unless otherwise stated. 100% CuBG scaffolds have shown considerably increased angiogenesis activity. Surprisingly, these series of scaffolds have been vascularized even more than VEGF-loaded control. CAM, Chorioallantois membrane; VEGF, vascular endothelial growth factor. Source: Reproduced with the permission from Ryan EJ, et al., Collagen scaffolds functionalised with copper-eluting bioactive glass reduce infection and enhance osteogenesis and angiogenesis both in vitro and in vivo. *Biomaterials* 2019;197:405–16. ©2019 Elsevier.

18.4.1.6 Zinc-containing bioactive glasses

In recent years, zinc (Zn) has been greatly intended as an important constituent of bioactive glasses. Zinc proves to be a stimulating factor for protein synthesis procedure and also for numerous enzymes [100–102]. It has been revealed that the presence of Zn in bioactive glasses could promote their chemical and mechanical features in contact with biological conditions. Zinc ions have also slowed down the degradation rate of bioactive glass scaffolds [103–105]. It is worth mentioning that Zn ions could help the growth of osteoblast cells via saving the pH value of biological surroundings. The role of Zn ions in biological conditions has been greatly influenced by its concentration. In a research focused on Zn-containing bioactive glass–ceramics, Brauer et al. [106] have indicated that utilizing more than 400 μM zinc resulted in cell death, however, low concentrations of Zn ions increased mechanical properties of the host glass–ceramics.

18.4.1.7 Cobalt-containing bioactive glasses

Cobalt (Co) ions are critically important in tissue engineering applications, thanks to their anticancer and antimicrobial properties. Co ions incorporated into the network of

bioactive glasses could activate angiogenesis and osteogenesis in defected bone tissues through providing hypoxic conditions: the hypoxia-inducible factor-1 (HIF-1) in mesenchymal stem cells, and HIF- α target genes [25,76,107,108]. In point of biocompatibility, applied concentrations of cobalt ions have a determinative role. High concentrations of cobalt ions could have serious cytotoxic and genotoxic effects [109]. As a suitable host, bioactive glasses could be utilized for controlled release of cobalt ions into the targeted tissues. In the network of bioactive glasses, cobalt ions act as the network modifier at a low concentration (1 wt.%) and as the network former at high concentrations (higher than 5 wt.%) [107]. It has been reported that replacement of some modifiers with cobalt ions in the 45S5-based bioactive glasses and glass–ceramics positively affected physicochemical and mechanical properties [107,110,111].

Hoppe et al. [110] have recently fabricated 1393 bioactive glass derived scaffolds through foam replica method. In order to stimulate angiogenesis and provide hypoxic conditions, they incorporated different concentrations (1 and 5 wt.%) of cobalt into the chemical composition of 1393 bioactive glasses (53SiO₂, 6Na₂O, 12K₂O, 5MgO, 20CaO, and 4P₂O₅, in wt.%). They have examined in vitro cell biocompatibility of the prepared scaffolds with osteoblast-like cells (MG-63) and human dermal microvascular endothelial cells (hDMECs). The reference glass doped with 1 wt.% CoO showed appropriate biocompatibility, in terms of cell viability, cell number, and morphology of osteoblast-like cells. However, 5 wt.% CoO in the glass composition induced considerable cytotoxicity. Fig. 18.9 shows the SEM micrographs and florescence images of MG-63 cells grown on different scaffolds after 14 days. In the case of 1393 and 1393-1Co scaffolds, cell adhesion and spreading is clearly detectable and the scaffold surface is totally covered by cells. However, cell spreading could not be detected on the 1393-5Co, which could be attributed to the cytotoxic effects (Table 18.1).

18.4.2 Silanization

Recently, silanization method has been extensively considered for increasing the physicochemical characteristics of biomaterials, owing to the presence of rich sources of OH groups on their surface [137,138]. The physicochemical characteristics of the bioactive glass surface can help modify surface physicochemical properties and the biocompatibility, arising from high content of OH groups on the surface of implanted material [139]. This method increases the interactions at the interface between biomaterials and biomolecules, which leads to dispersion stability of biomaterials in the biological fluids and improvement in the drugs' immobilization [140]. It has been proven that the silanol groups of hydrolyzed silicon alkoxides having functional groups of –NH₂, –Cl, –COOH could react with hydroxyl groups of the biomaterial's surface. As it was shown earlier, cell responses to the protein adsorption of biomaterials is an important parameter in biological cell response behavior, having a positive role on the protein adsorption on the biomaterials' surface [141]. One of the well-known silanes named 3-aminopropyltriethoxysilane (APTES) has been reported by researchers for improvement of the surface characteristics of bioactive glasses without reducing its bioactivity. In a research conducted by Lu et al. [142], the surface of bioactive glasses was treated by APTES. The Fourier-transform infrared spectroscopy spectrum taken from the treated sample confirmed the presence of saturated

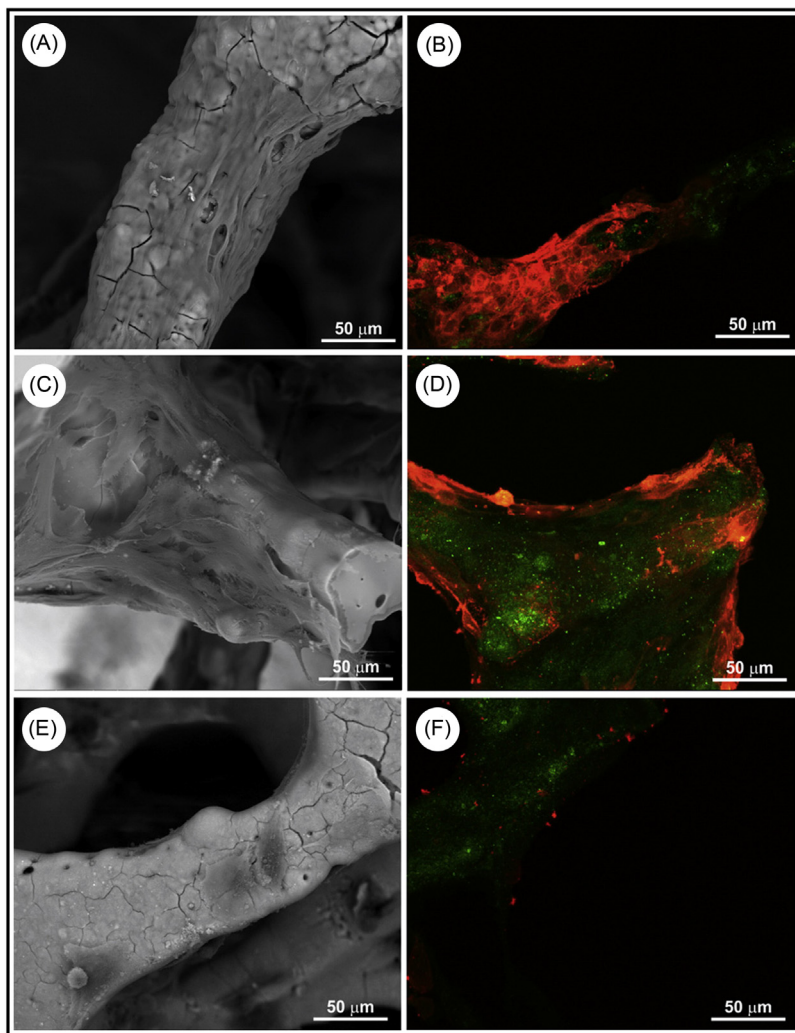


FIGURE 18.9 SEM images (A, C, and E) and fluorescence images (B, D, and F) of osteoblast-like cells seeded on 1393 (A and B), 1393-1 wt.% Co (C and D) and 1393-5 wt.% Co (E and F) bioactive glass scaffolds. Red shows Vybrant (cytoskeleton)-stained cells and green is auto fluorescence of BG surface. SEM and florescence images were respectively taken by scanning electron microscopy and confocal laser scanning microscopy. Source: Reproduced with the permission from Hoppe A, et al., *In vitro cell response to Co-containing 1393 bioactive glass*. *Mater Sci Eng C* 2015;57:157–63. ©2019 Elsevier.

C–H bonds with stretching vibration. On the other hand, differential thermogravimetry curve indicated an extensive mass loss referring to combustion of the organic group and indicating the interactions between the bioactive glass surface and the APTSE. Controversially, some researchers showed that the MC-3T3 cell growth on silane-treated sample was extensively higher than untreated sample. In addition, some scientists designed a novel mesoporous bioactive glass having amino-functionalized groups by using APTES implant with high drug load capacity and extended drug release. It has been reported that the surface area of silane-treated samples having amino groups was extremely increased compared to the untreated sample [143]. According to the in vitro bio-activity assessment, the treated samples were proven to be desirable for biological applications.

TABLE 18.1 The effects of different therapeutic ions on the biological responses of bioactive glass derived scaffolds.

Ion	Biological responses	References
Fluoride	• Dose dependency of the cell responses • Appropriate biocompatibility in low concentrations • Higher compatibility of sodium free bioactive glass derived scaffolds compared to sodium containing scaffolds	[112–116]
Magnesium	There is a contradiction about cell responses to magnesium: • Better cytocompatibility of scaffolds containing 5 wt.% magnesium in comparison with those having higher concentrations • Better cytocompatibility of scaffolds containing 10 wt.% magnesium in the host bioactive glass	[117–119]
Strontium	• Inhibition of cell viability at high concentration (about 15 mol.%) • Biocompatibility at different particle size of the host bioactive glass	[6,76,120–126]
Silver	• Dose dependency of cytotoxicity (cytotoxicity occurs at concentrations higher than 1 wt.%)	[127–129]
Copper	• Dose dependency of the cell responses • Appropriate biocompatibility at low concentrations (below 10 mg/L)	[130–132]
Zinc	• Cytotoxicity effects at high concentrations (higher than 0.5 wt.%)	[133–135]
Cobalt	• Cytotoxicity effects at high concentrations (higher than 1 wt.%)	[25,76,136]

18.4.3 Surface functionalization of bioactive glasses through biological approaches

As it was shown earlier, cell responses to the protein adsorption of biomaterials are an important parameter in biological cell response behavior. It has been proven that the proteins' adsorption is influenced by the substrate's chemical composition. Recently, immobilization of biological molecules and cells through physical or chemical approaches on the surface of bioactive glass seem to be promising tools to improve and modify the adsorption of proteins on the glass surface. As previously described, another example of chemical immobilization is silanization. Besides, surface of bioactive glasses could be functionalized to increase the bone integration. The protein adsorption on the bioactive glass's surface could considerably affect the formation of HCA-like layer on the bioactive glass surface, in different ways [26,144,145]. Some studies have shown that the cell spreading on the surface of bioactive glass was just about 5% in the absence of FN protein, while this amount became 100% in the presence of this protein [146]. Nevertheless, the HA-like layer formation on the surface of bioactive glasses could be postponed by some serum proteins. Besides, the protein receptors in proximity of the surface of bioactive glass could increase surface biocompatibility [26]. Furthermore, calcium phosphate formation on the surface could extensively increase surface protein adsorption of bioactive glasses [147]. It has been reported that the proteins adsorbed on the modified surfaces had an extensively higher concentration compared to the unmodified samples [148].

18.5 Commercialized bioactive glasses and glass–ceramics

Since more than 30 years ago, Hench's original 45S5 Bioglass has been applied in about 1.5 million clinical trials for applications of bone tissue engineering [12,149,150]. Replacement of the small bones of the middle ear was clinically applied by implantation the first 45S5 Bioglass in the United States [151]. This product later received FDA approval under the commercial name of "Bioglass Ossicular Reconstruction Prosthesis" or "Middle Ear Prosthesis" MEP [152]. MEP showed better efficacy by short/midterm results compared to nearly inert scaffold [153]. Application of this class of biomaterials in the form of long-term clinical usage was accompanied by vast dissolution in the body. Consequently, a few years later, these implants were all taken from the US market. Nevertheless, Douek-MEDTM, that is, a modified version of those implants, is still popular in the market of some countries [154]. Ceravital glass–ceramics, that is, a crystallized type of glass-based scaffold, have been used as the implant of the middle ear's small bones for a long period of time; but recently it was banned from the market due to dissolution in the biological milieu overtime [155]. Another commercialized bioglass-based scaffold is Bioglass-EPI (extra-cochlear percutaneous implant) which is applied for fixing cochlear implants to the temporal bone of seriously deaf patients [156]. After a few years, the Bioglass-EPI implant has been also banned from the market due to the dissolution in the biological environment. Afterwards, another dental implant named Endosseous Ridge Maintenance Implant (ERMI) has been retailed in the market, especially for dental regeneration applications [157]. After 5 years of experimental and clinical examination of this implant, the results confirmed the high safety and stability. Nonetheless, their extensive applications have been limited due to their rigidity and inflexibility [158]. Some long-running clinical studies showed that custom-made monolithic bioactive glasses were more suitable than these mentioned scaffolds for orbital bone fractures treatment [159]. Besides, particles of bioactive glasses, for example, PerioGlas (NovaBone Products LLC, Alachua, Florida, United States) have been on retailed in the market due to high flexibility to be shaped into various bone defects, mainly jaw and tooth. Furthermore, another commercialized implant under the name of Biogran (Biomet 3i, Palm Beach Gardens, Florida, United States) has been utilized for jaw bone regeneration having narrower particle size distribution in comparison with PerioGlas [160]. Moreover, many other bioactive glass implants have been also commercialized in the market [161]. Most of the newly introduced bioactive glasses were silicate based with some modification of chemical composition [162]. These included 3-93 ($53\text{SiO}_2-6\text{Na}_2\text{O}-12\text{K}_2\text{O}-5\text{MgO}-20\text{CaO}-4\text{P}_2\text{O}_5$, in wt.%) and S53P4 bioactive glasses, which proved to have realm of possibility in the process of bone regeneration. Another commercial product under the name of BoneAlive proved to accelerate bone regeneration process in comparison with 13-93, thanks to the presence of MgO. Some sol–gel processed bioactive glass powders such as TheraGlass, MedCell, Burgess Hill, and UK accelerated bone regeneration process due to their intrinsic nanoporosity, compared to the melt-derived products. Other than that, 45S5-based implants have been commercialized for oral regeneration [163,164]. Previously, NovaMin (NovaMin Technology, Florida, United States; now owned by GlaxoSmithKline, Brentford, United Kingdom), a modified 45S5 Bioglass particulate was supplied in toothpaste for dental hypersensitivity treatment.

Some of the commercial bioactive glass products could be utilized in soft and hard tissues due to their angiogenesis functions [165,166]. For instance, bioactive glass nanofibers (basic 13-93B3 with chemical composition as $53\text{B}_2\text{O}_3-6\text{Na}_2\text{O}-12\text{K}_2\text{O}-5\text{MgO}-20\text{CaO}-4\text{P}_2\text{O}_5$, in wt.%), under the trade name of DermaFuse/Mirrugen could likely enhance the regeneration of long-term venous stasis ulcers in diabetic patients [167,168]. Another commercial bioactive glass material known as “RediHeal” (Avalon Medical, Stillwater, Minnesota, United States) has exhibited possible angiogenesis functions due to the presence of doped copper in its formula. As described above, much research has been carried out considering doping various ions inside the bioactive glass composition. This research subject is very challenging and will continue to open new opportunities in the market of regenerative medicine and tissue engineering.

18.6 Discussion

Bioactive glasses and glass–ceramics have been growing in recent years for application in regenerative medicine and tissue engineering. Contrary of their progressive biomedical applications, there are still some unsolved issues, especially related to mechanical strength and biocompatibility of these materials. Among bioactive glasses and glass–ceramics, silicate-based materials have been more explored rather than borate and phosphate-based ones. Therefore other classes of bioactive glasses should be more explored to be targeted for tissue engineering applications.

Newly proposed bioactive glasses also present angiogenesis activity which is vital for reconstruction of soft tissues. However, in order to guarantee these materials for soft tissue engineering applications, much more *in vitro* and *in vivo* examinations should be done. In the past two decades, several strategies have been focused on functionalizing bioactive glasses, concerning their bioactivity, biocompatibility, and antibacterial effects. In this regard, several therapeutic metallic ions have been doped into the network of the host bioactive glasses. On the basis of numerous researches, dose-dependent efficacy of the incorporated ions proved to be a critical issue, in point of cytotoxicity. Therefore toxicity of each dopant should be examined both *in vitro* and *in vivo* before clinical applications. Take apart from biological features of bioactive glasses, improvement of mechanical properties should be considered in future research. It has been proposed to utilize bioactive glasses in the form of composite materials to preserve biomechanical properties. For example, it is possible to design zirconia or alumina/bioactive glass composites with improved biomechanical properties [169,170]. However, this newly introduced strategy needs more investigation.

Moreover, further research should be conducted in such a way that elucidates all factors which are involved in biological responses to bioactive glasses. In this regard, adsorption of proteins on the surface of bioactive glasses and their impacts on the biological response of surrounded tissues should be deeply explored. On the other hand, as reported by Shah et al. [171], the condition of biological medium such as composition and pH, could change the biological response to the implanted bioactive glasses. Therefore exact characterization of biological media is critically important.

These days, many researchers are working on bioactive glasses to have a better understanding of different mechanisms that are involved in cellular and molecular responses to this outstanding class of biomaterials. In the very near future, it is expected that different types of bioactive glass and glass–ceramic materials with different chemical compositions and forms act as the key role players for both hard and soft tissue engineering applications.

18.7 Conclusion

During the last few decades, there has been considerable interest towards bioactive glasses and glass–ceramics as high potential biomaterials for biomedical applications owing to their ability of chemical interactions with adjacent tissues. In this regard, many researchers have investigated the biological responses to bioactive glasses and glass–ceramics as proper biomaterials for application in tissue engineering. In this context, various modifiers such as ions and additives have been applied to improve the cell signaling pathways. It seems necessary to provide adequate knowledge on the explicit cell signaling performance of this category of biomaterials. However, there is still much to be done to ascertain the biological responses to the bioactive glasses and glass–ceramics and more particular research should be performed in this field. The literature yet lacks a legitimate and proper information database in terms of the specific mechanisms participating in the FBRs, which should be considered as well.

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Cell responses to titanium and titanium alloys

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19.1 Introduction

Commercially pure titanium and titanium alloys (Ti) are considered as gold standards for skeletal biomaterials for designing dental and orthopedic implants, attributed to their favorable mechanical properties and biocompatibility [1–4]. These include high specific strength, high fracture toughness, and low elastic modulus. The latter reduces the “stress shielding” effect in peri-implant bone and thus, minimizes bone resorption. The instantaneous formation of a passive and stable oxide layer on the surface of Ti makes these materials highly resistant to corrosion and biocompatible for the aforementioned biomedical applications. Ti combines physicomachanical and bioinert properties that are more favorable for dental and orthopedic applications than other metals like cobalt, nickel, chromium, and their alloys [5].

The use of Ti for implant fabrication dates back to the 1930s when its lightweight and good mechanochemical properties made it the material of choice over stainless steel and cobalt-alloys, which were then commonly used implant materials. Since then, Ti has found application in dentistry and craniofacial surgery for fabricating implants, crowns, or other prosthesis [6], but also in orthopedics for hip and joint replacement and spinal fixation devices [7]. The most commonly used forms of Ti for implant fabrication include different grades of commercially pure titanium (CP-Ti) and the extra-low interstitial titanium alloy, Ti6Al4V. The superelastic and shape memory alloy, Nitinol (Ni–Ti) has been also used in the biomedical field for manufacturing guided wires, orthodontic braces, heart valves, and fixators for fractured bones to name a few [8,9]. Limitations associated with toxic ions released from alloys or mismatch in the mechanical properties of Ti or Ti-alloys have stimulated extensive research in designing different alloys. The most common alloying elements that have been incorporated to overcome the associated issues include Nb, Zr,

Mo, Sn, and Ta, which are known to reduce the modulus of elasticity of Ti [10–12]. Another approach for tailoring the mechanical properties of Ti is 3D-printing porous materials using additive manufacturing (AM), selective laser melting, and electron beam melting [12–14]. AM printed implants not only offer the capability of manufacturing mechanically matched porous implants by tuning the structure design but can also be personalized to be patient specific.

Irrespective of the bulk material properties of Ti and the design and manufacturing process, these materials are bioinert, which is a surface-related property. The bioinert nature of Ti, which was historically considered a suitable avenue for using Ti as implanted biomaterials, has been addressed over the last 15–20 years, so that Ti can display bioactive properties. As Ti are bioinert, they do not elicit any specific favorable biological response when they come in contact with environmental biological agents, such as water, proteins, cells, or bacteria. As a consequence, the healing and integration of Ti in peri-implant tissues are significantly delayed and/or the Ti implant gets infected. To address one or both the issues, research has been focused on different surface modification techniques in Ti to enable favorable cell responses and/or fight bacteria [15].

This chapter highlights cellular responses to Ti surfaces modified with different engineering strategies; primarily those (1) for inducing favorable cell behavior on Ti implants for replacement or regeneration of bone, soft tissue, and control of host immunological response; and (2) for preventing peri-implant infection. Some of the strategies to control biological Ti surface properties include physical modification, which alters surface topography and roughness, and other strategies are focused on chemical modification, which includes inorganic and organic coatings. Understanding the effect of each of these surface properties on cellular response in vitro can guide researchers in designing an implant with combinatorial surface characteristics to elicit a favorable response in vivo.

19.2 Surface modification of titanium alloys to induce appropriate cell responses

19.2.1 Repair and regeneration of hard tissues

19.2.1.1 *Surface topography and surface roughness*

The role of surface topography in controlling the fate of cells was illustrated as early as 1911 by Harrison, where the cells aligned themselves along the length of spider web fibers [16]. Since then lot of effort has been made to understand the relevance of surface topographical cues in dictating cell behavior. Surface topography (and thereby surface roughness) has been well identified to regulate indirect cellular mechanotransduction responses, which are mediated by modulation of integrin clustering and cell adhesion to the substrate [17,18]. In vitro studies assessing the influence of surfaces featuring micron-scale topography have been studied extensively. The consensus is that rough surfaces support MG-63 osteosarcoma cell line proliferation and differentiation with reduced initial attachment levels [19,20]. Surface roughness also influences “adhesion power” (AP) a parameter used to assess long term adhesion of cells on surfaces with different roughness [21]. Long term culture periods of 21 days indicated fusion of cells with no distinct visible boundaries on

smooth surfaces and low AP, whereas rough surfaces supported extracellular matrix (ECM) deposition and high AP. Surface roughness is also known to promote the influence of exogenous factors like vitamin D or 17β -estradiol in stimulating a differentiated phenotype of MG-63 when compared to flat surfaces [22,23]. Presence of anisotropic features with micron-scale roughness is also known to support higher cell adhesion and proliferation when compared to isotropic rough surfaces, which supports contact guidance [24]. Whereas rough surfaces support mesenchymal stromal cells [25], osteoprogenitor cells and osteoblasts [26–29] adhesion, proliferation and differentiation; cells from soft-tissue sources like fibroblasts notably adhere and proliferate on relatively smooth surfaces [30]. Several reviews have highlighted the correlation of surface microtopography and cell response in vitro and subsequently in vivo [20,24,31,32].

With the advancement in technologies for surface modification and the understanding that the cells respond to the organization of ECM, which happens at the nanometer scale, as illustrated in Fig. 19.1, there has been a gradual drift from using classical techniques to produce microrough surface features to advanced techniques to produce controlled nano-featured/textured surfaces [33,34]. There is also motivation for combining techniques, which is driven from the goal of incorporating diverse topographical features of the hierarchical structure of bone, ranging from micrometer scale down to nanometer scale. This is done to mimic bone characteristics during bone remodeling which includes formation of micron-sized pits with submicron scale roughness due to osteoclast activity and the nano-scale features contributed from the collagen fibers left after bone resorption [35]. The nanostructured surfaces are reported to favor cell adhesion, proliferation and cell differentiation [36–42].

Different surface engineering techniques can be used to obtain nanoscale features, like electrochemical anodization, template-based method, lithography and contact printing [43]. Anodization and template-assisted method can be used to fabricate uniform nanostructures. Electrochemical anodization is commonly employed to form an array of nanoporous

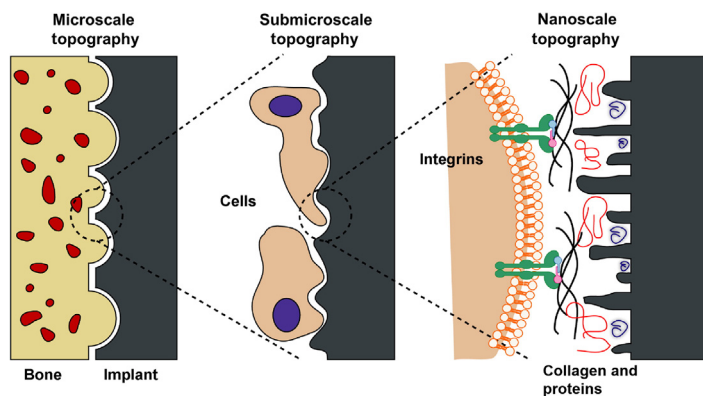


FIGURE 19.1 Illustration of interactions between bone cells and an implant surface at the microscale ($1\ \mu\text{m} \leq \text{dimension} < 1000\ \mu\text{m}$), submicroscale ($100\ \text{nm} < \text{dimension} < 1\ \mu\text{m}$) and nanoscale (dimension $\leq 100\ \text{nm}$) [35]. Source: Reprinted from Gittens RA, et al. The effects of combined micron-/submicron-scale surface roughness and nano-scale features on cell proliferation and differentiation. *Biomaterials* 2011;32:3395–403 with permission from Elsevier.

structures on the surface, commonly termed as nanotubes. By changing the electrolyte and the anodization potential, the physical properties of the nanotube array can be tailored resulting in nanotubes with different pore diameters and lengths. The nanotube modified surfaces have shown to promote osteogenesis both in vitro and in vivo when compared to their unmodified counterparts [44–48]. Hydrothermal method is another way of fabricating nanotubes on titanium-based surfaces and is relatively cheaper compared to electrochemical anodization, which can be translated to complex 3D implants [49]. By tuning the solvent used (water or sodium hydroxide or hydrogen peroxide) and the treatment temperature, nanostructure shape can be easily varied from leaf-like to pyramid-based structures [50], nanorods [51], and nanotubes [52]. Hierarchical structures at different length scales (nano/micro/macro) on titanium alloy prepared by this technique demonstrated improved mesenchymal stem cell adhesion, proliferation and differentiation compared to the untreated controls [53]. By carefully tuning the nanotopology on the material achieved by any surface modification technique, specific bioactive response can be achieved which can thereby allow improved osseointegration and survivorship with minimized revision surgery requirements when compared to flat controls [33].

19.2.1.2 Surface wettability and free energy

Surface wettability and free energy plays a critical role in conjunction with other material interface properties in determining the nature of the protein layer and its downstream effect on cell function. Surface energy is defined as extra energy that the interface has in comparison to the bulk which thereby drives the adhesion of surrounding cells. Wettability on the other hand describes the interactions between the liquid and adjacent solid interface [54]. These parameters play a critical role especially in cases where two surfaces look comparable in chemical composition but show different biological response. Different approaches have been tested to alter surface free energy and the downstream effect of this surface interfacial property on protein adsorption and cell response. Irrespective of the approach used, increased protein adsorption [55,56] and upregulated osteogenic differentiation in vitro [57] and bone integration in vivo [58] has been reported on surfaces with high surface energy compared to the untreated controls. With increased surface energy there is an increase in surface wettability which enhances surface interaction with the surrounding biological environment [59,60]. It is proposed that changes in cell differentiation are mediated via altered integrin expression as an outcome of altered surface wettability [61]. Increased wettability of the surface is known to promote cell maturation and differentiation both in vitro and in vivo [25,62–65].

By carefully adjusting surface free energy and thereby wettability, differences in the biomolecular conditioning can be achieved. This is manifested in differences observed in nature, conformation and the strength of proteins adsorbed on the surface. These differences are known to dictate the cellular response elicited at the interface, where high levels of cell adhesion, cell spreading and cell differentiation of hard-tissue cells are observed on hydrophilic surfaces and none on the hydrophobic surfaces. Similar responses are observed for cells derived from soft-tissue (keratinocytes and fibroblasts) where improved wettability supports cell adhesion and proliferation [66]. These responses are not only limited to hard and soft tissue derived cells but also induce macrophage activation to M2-like state producing an anti-inflammatory environment [67]. The consensus in the literature

seems to point out to a stimulating effect of surface wettability on hard and soft-tissue derived cells *in vitro*, and improved healing and integration *in vivo*. However, this topic still needs to be soundly resolved with further studies to elucidate different effects of these physical–chemical properties on different types of cells as well as the correlations between the effects on cells *in vitro* and *in vivo*.

19.2.1.3 Surface chemistry

To overcome the risk of delayed integration of Ti implant in bone and/or eventual implant failure, different chemical based modification strategies have been employed. This is done to impart bioactivity to relatively inert titanium and titanium alloys. Most of these surface modifications derive inspiration from incorporating biominerals, mimicking biomineralization processes, and/or inducing the formation of hydroxyapatite (HA) *in vivo* to upregulate interfacial bone formation between the implant and the surrounding tissue [68]. These approaches can be broadly classified as coating approaches, namely, inorganic coating which relies on forming bioactive oxide layer or calcium phosphate based coating and organic coatings which comprises of modifying the surface with ECM molecules that can mediate either mineralization of the surface or appropriate direct interactions with proteins and/or cells.

Inorganic coatings

Earlier efforts of chemical modification of Ti surfaces involved utilizing the sol–gel technique to form a stable oxide layer on the surface of Ti, which would accelerate the osseointegration process *in vivo* and enhance the interfacial bone-implant bond. These modifications were in succession of hydrogen peroxide treatment of titanium, which was reported to form a biocompatible titanium-peroxy gel and support osseointegration. These modifications often resulted in formation of calcium phosphates within the hydrated surface film in simulated body fluid, enhanced cell adhesion, proliferation, and differentiation *in vitro* and a strong interfacial connection with the surrounding bone *in vivo* [69,70]. Acid and alkali treatments are other commonly used surface modification approaches applied on Ti which are known to confer bioactivity by improving cellular interactions and/or inducing the formation of calcium phosphates on the surface of Ti. Acid treatment normally utilizes strong acids like hydrochloric acid, sulfuric acid, nitric acid, and hydrofluoric acid either by themselves or in combination with other acids. These treatments remove surface contaminants and result in the formation of ~10 nm thin oxide layer. Ti surfaces treated by acid etching are known to favor osteogenic gene expression in several *in vitro* studies and improved osseointegration *in vivo* [25,71,72]. Similar to acids, alkalis like sodium and potassium hydroxide are also used to treat titanium and its alloys resulting in the formation of surface titanates which can trigger the nucleation of calcium phosphates in simulated body fluids *in vitro* as well as *in vivo* after implantation. The chemically modified surfaces demonstrated enhanced *in vitro* apatite formation but also stimulated cell attachment, proliferation, and gene expression of osteogenic markers [73–76]. Alkali treatment is used in conjunction with heat treatment which results in a porous surface layer that is reported to stimulate osteogenic differentiation of MSCs but also showed maximum bone bonding strength [69]. Alkaline etching on Ti has also been combined with microroughness by short blasting, which accelerates calcium phosphate

formation *in vitro* [77] and rate of osseointegration *in vivo* [78–80]. Different univalent [silver (Ag), lithium (Li)], and divalent/trivalent [magnesium (Mg), strontium (Sr), gallium (Ga)] ions, to name a few, have been reportedly incorporated in the surface with improved functionality [81–84]. Doped titanates are reported to demonstrate significant improvements in biological response in terms of osteogenic differentiation of cells *in vitro* but are also known to improve bone implant interface *in vivo* [85,86].

Another common chemical modification utilizes the inorganic constituent of bone. This includes direct coating of Ti surfaces with calcium phosphates. The most extensively used bioactive calcium phosphate is HA, as it is the mineral in bone. HA-coated Ti results in improved biocompatibility, osteoconductivity, and bone healing process when compared to Ti controls [31,87–89]. The bioactive nature of HA coatings is in part attributed to the adsorption of large amounts of fibronectin and vitronectin on HA, which are key proteins mediating cell adhesion and proliferation [90]. Also, HA coatings can stimulate apatite formation and precipitation of bone morphogenic protein (BMP) 2, which plays an important role in recruiting cells to undergo osteogenic differentiation [91,92]. The application of HA bioactive coating on Ti implants via plasma spraying in the 1980s was the tipping point where the strategies applied for surface modification were not to have a protective effect but instead played an active role in orchestrating a desired biological response [93]. Since then different aspects of the deposited coatings like Ca/P ratio, phase composition, and nature of crystallinity in conjunction with different coating techniques have been extensively tested for their biological performance. Different coating techniques for preparing HA coatings on metal substrates have been consolidated for obtaining strongly adherent, uniform, and reproducible coatings [94,95]. Alongside, the last two decades have witnessed a push for doping the deposited HA coating with different ionic species. Silicon (Si)-doped HA [96–99], strontium (Sr)-doped HA [100–102], magnesium (Mg)-doped HA [103,104], silver (Ag)-doped HA [105,106], fluorinated (F)- [107,108] and carbonated (CO₃)-doped HA [109,110] have been developed. Most of these dopants get incorporated into the crystal lattice replacing either the Ca or PO₄³⁻ or OH¹⁻ and alter the stability of the coating. Si, Sr, and Mg doped coatings have been reported to support cell proliferation and differentiation when compared to CaP coatings. Sr dopant is also known to reduce osteoclast proliferation whereas Ag is known to render antibacterial properties to the coated surface [111].

Another ceramic that has found utilization for titanium surface modification includes bioactive glass, discovered by Hench et al. in the late 1960s. These constitute a class of bioactive materials that are synthetic, degradable ceramics, and have demonstrated the ability to form a strong interfacial bond with the surrounding tissue [112]. Enhanced osteogenesis is associated with the ability of these bioactive glasses to stimulate the formation of a biologically active layer of hydroxycarbonate apatite (HCA). The formation of HCA layer is mediated via exchange of Ca²⁺, Mg²⁺, and Na¹⁺ ions with environmental H¹⁺ ions and hydrolysis of Si–O–Si bonds resulting in the formation of silica (Si–OH) gel at the interface. Poor mechanical properties and intrinsic brittle nature of these ceramics limits their usage in load-bearing conditions which prompts their application as coatings to harness the bioactive nature of these materials [113]. Different coating strategies have been tested including plasma spraying, electrophoretic deposition, sol–gel method, RF magnetron sputtering, to name a few. However, limited success has been achieved due to poor

adhesion of the coatings and mismatch of thermal expansion coefficient of the coating and the underlying substrate to which the coating is applied [114]. These limitations along with long-term stability of the coating in vivo has hindered the use of these coatings in commercial setting and therefore optimization of the composition of the bioactive glasses and the coating strategies is required to harness the bioactive potential of the material to the fullest.

In some cases hybrid coatings of TiO_2/CaP are prepared on titanium and titanium alloys. This is done to exploit the potential for strong adhesion of the TiO_2 layer with the underlying substrate, which then is used as a mechanical buffer layer for the preparation of the bioactive CaP coating. These composite coatings support osteogenic differentiation of cells in vitro and improved bone-implant interfacial strength in vivo [115].

Organic coatings

The last few decades have seen a transition from using inorganic ceramic-based coatings to utilizing organic coating which are either natural or synthetic polymers or derivatives of ECM. Natural or synthetic polymers offer the capability to alter surface wettability and surface degradability whereas ECM-derived proteins and peptides closely mimic the natural substrates and interfaces of the cells [116]. This is in part motivated to make the surface more viable for cell adhesion and downstream function to accelerate tissue repair/regeneration [117]. These include modifying surfaces with different synthetic or natural polymers, peptides, proteins, and polysaccharides to dictate the response elicited at the implant–tissue interface.

Natural and synthetic polymers, as mentioned earlier are commonly employed to provide an interface with prespecified properties that facilitate tissue remodeling which happens in conjunction with polymer degradation without releasing toxic byproducts. The most extensively investigated synthetic polymers include degradable materials like poly(D, L-lactic-co-glycolic acid), polycaprolactone, or hydrophilic biostable polymers like polyethylene glycol and poly(vinyl alcohol) [4]. Efforts have been extended to design smart coatings that deliver the payload under defined conditions. One such example includes utilizing stimuli responsive coating based on polydopamine/polypyrrole microcapsule system loaded with dexamethasone onto titanium substrates. The microcapsules were capable to demonstrate triggered release of dexamethasone in the presence of electrical stimuli and showed improved cell adhesion, proliferation, and alkaline phosphatase (ALP) activity of bone marrow stromal cells along with good biocompatibility in vivo [118]. This might be extended to a series of polymers loaded with an array of biological and chemical factors with different degradation rates so that temporal control of factor release is controlled.

Amongst natural polysaccharides, chitosan has been extensively utilized due to its biocompatibility, biodegradability, antimicrobial, and wound healing properties. Chitosan-coated surfaces are known to support bone and cartilage tissue growth due to its structural similarity to glycosaminoglycans. Chitosan, however, has poor solubility and mechanical properties, and to overcome this limitation, it is chemically modified or mixed with PVA or alginate or pectin to form composite coatings which have shown improved mechanical properties with elastic modulus comparable to bone and cell response [119,120].

Inspired from elastin, an elastic protein present extracellularly in large vertebrates, elastin-like recombinamer (ELR) has drawn significant attention for biomimetic modification of Ti. Tethering ELRs linked with RGD (arginine–glycine–aspartic acid) sequences covalently onto titanium are known to have antifouling properties and support MSC adhesion and differentiation relative to the control [121]. Mineralized ELR-HSS (peptide derived from human salivary statherin)-coated Ti resulted in a hybrid nanorough surface with enhanced adhesion and differentiation of preosteoblasts compared to the noncoated Ti [122].

Coating surfaces with cell adhesive proteins, like collagen, fibronectin, or vitronectin, are known to improve cell adhesion, proliferation, survival, and migration [123,124]. These coatings can be patient specific and tuned to incorporate selected biochemical cues that can accelerate osseointegration of Ti in vivo [124]. Collagen type I, which constitutes 90% of the bone ECM, is one of the extensively studied proteins for coating implant surfaces. This is partly attributed to its biodegradability and low antigenicity, which makes collagen an ideal choice as a bioactive coating. These coatings showed no cytotoxicity in vitro [125–127] and enhanced bone growth and long term stability in vivo [116,128–130]. BMPs and transforming growth factor β (TGF- β) which belong to TGF- β superfamily constitute a secreted protein superfamily which plays an important role in bone formation and disruption in the TGF- β /BMP signaling results in bone diseases like osteoarthritis and tumor metastasis [131]. These proteins have been utilized as coatings on titanium implants which thereby significantly enhance new bone formation by mimicking the natural environment of the bone-forming cells [132,133]. TGF- β coatings on Ti surface can also reduce cell responses that trigger the formation of fibrotic tissue as an alternative approach to enhance osseointegration of Ti surfaces [134]. Therefore combining the collagenous and noncollagenous bone matrix proteins as coating matrices could significantly enhance new peri-implant bone formation in vivo.

Short peptide sequences have been utilized to activate the surface as an alternative to large protein molecules as these sequences are known to retain cell interaction's activation from those of whole proteins. The RGD peptide is the most extensively studied peptide sequence for enhancing cell adhesion to synthetic substrates, including Ti surfaces [135,136]. Interaction of the immobilized RGD peptides with the cytoskeletal integrin receptors activates different signaling pathways, which thereby alter cell architecture and/or stimulate cell proliferation. Coimmobilizing RGD peptide with PHSRN peptide utilizing silane chemistry supported cell adhesion, proliferation, and differentiation when compared to surfaces immobilized with single peptide [117]. RGD and PHSRN are present adjacently on type III repeating unit of fibronectin, which is known to activate cellular integrins [137,138].

Another class of peptides that have gained recent interest is chimeric peptides. Modifying surfaces with these chimeric/fusion peptides allows cell-specific responses which when combined with surface adhesion peptides allows tethering of the peptide to the biomaterial surface [139,140]. These peptides support homogenous presentation of cell binding sequence to support a cell response directed toward a specific cell population without affecting other cell types. Modifying apatite surface with DPI (MSC specific)-VTK (mineral binding) dual peptide recruited bone-forming progenitors from heterogeneous progenitor cell population and supports MSC adhesion, proliferation, and differentiation

on apatite surface. This approach has been extended to 3D porous polymeric scaffolds and results demonstrate uniform adhesion and proliferation of induced pluripotent stem cell-derived MSCs with increased vascularized bone formation *in vivo* [141].

Along with applying inorganic or organic coatings separately and assessing the biological response, composite coatings have also been assessed. The most common form of composite coating tested includes CaP and collagen coating which has been reported to enhance cell adhesion, proliferation, and subsequent cell differentiation [24,128]. Collagen coating not only provides the necessary RGD sequences that can interact with integrin receptors but also serves as a reservoir for CaP crystals [142]. The controlled release is not limited to CaP crystals but has also been extended to growth factors like TGF- β , BMP2, where the sustained release of collagen immobilized growth factors showed increased osteogenic properties when compared to growth factors immobilized on untreated titanium surfaces [143–145]. Recent strategies include utilizing osteoinductive signaling factors which can help in recruiting cells to the implant by forming a chemoattractive factor gradient. One of the most commonly used cytokines is stromal cell-derived factor 1 α (also called CXCL12) which when used in conjunction with BMP2 significantly improved new bone formation [146].

19.2.2 Repair and attachment of soft tissue

Healthy soft tissue healing and attachment to implants plays a key role in the success of percutaneous implants, either dental implants and/or osseointegrated transdermal devices. The ability of the implant material to support formation of a biological seal which can then prevent premigration and postimplantation infection is critical for its long-term clinical success [147–149]. As in the case of interactions of Ti surfaces with bone, excellent mechanical strength, and biocompatibility of Ti enables use of this material in transdermal applications. However, again, the bioinert nature of Ti makes it incompetent to stimulate the formation of an impervious seal with the surrounding soft tissue. Deriving inspiration from the different nanostructures present in skin, surface modification of Ti have been tried to enhance skin-implant integration. Nanotextured surfaces prepared by electron beam evaporation supported keratinocyte adhesion and proliferation complimented with extended filopodia [150]. There is, general consensus in that there are no significant beneficial effects on keratinocyte responses when they interact with nanotubular surfaces [151], but enhanced proliferation and differentiation can be achieved when keratinocytes are cocultured with fibroblasts [152]. Fibroblasts on the other hand, are known to be positively influenced by a combination of micro- and nano-topography, which support cell adhesion and proliferation when compared to flat controls [153,154].

Several chemical modification strategies have also been employed to mimic the biological environment of the soft tissue to strengthen the interface between the biological tissue and Ti implant. One such strategy uses silane chemistry to immobilize peptides derived from the Laminin-332 protein and ameloblastin. These bipptide modified surfaces resulted in enhanced keratinocyte adhesion, early stage proliferation, differentiation, and *in vitro* hemidesmosome formation [155]. Combining microstructured porous titanium with adhesive peptide derived from another but similar laminin protein family (Laminin-5)

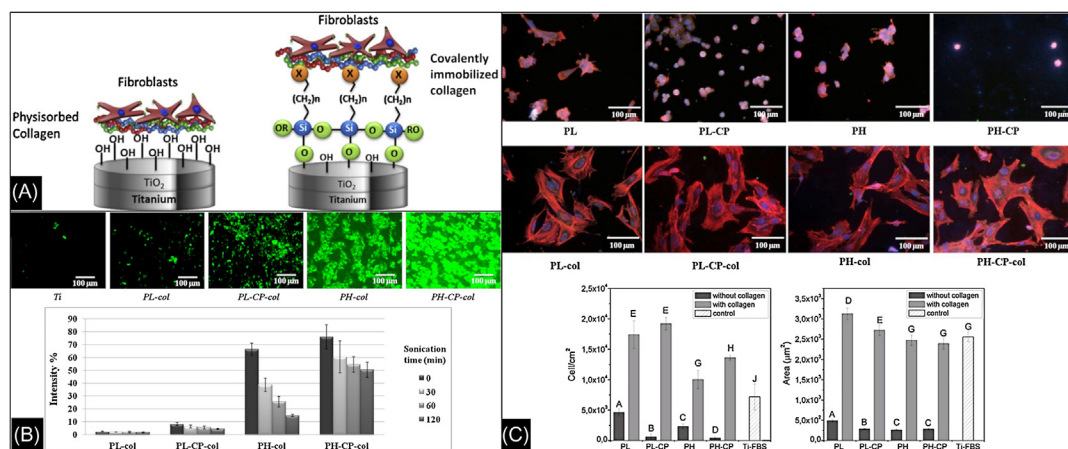


FIGURE 19.2 (A) Strategies for collagen immobilization on titanium surfaces and its subsequent effect on fibroblast response. (B) Visualisation of type I collagen by 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA) fluorescence and the graph represents the mechanical stability of type I collagen on different Ti surface after sonicating samples for different time periods, as determined by the optical intensity of fluorescence microscopy images. (C) Fluorescent images of the fibroblasts cultured for 4 h on different sample groups (top panel) and quantification of the cell number and cell area for the fibroblasts cultured for 4 h on different sample groups (bottom panel). Different sample groups analyzed include: Ti (commercially pure Ti), PL (oxygen plasma treated polished Ti), PL-CP (PL sample modified with 3-chloropropyl-(triethoxy)silane (CPTES)), PH (piranha treated polished Ti), PH-CP (PH sample modified with CPTES), PL-col (PL with physisorbed collagen), PL-CP-col (PL-CP sample with covalently immobilized collagen), PH-col (PH with physisorbed collagen) and PH-CP-col (PH-CP sample with covalently immobilized collagen [159]. Source: Reprinted from Marin-Pareja N, et al. Collagen-functionalised titanium surfaces for biological sealing of dental implants: effect of immobilisation process on fibroblasts response. *Colloids Surf, B: Biointerfaces* 2014;122:601–10, reproduced with permission from Elsevier.

improved cell adhesion in vitro and improved soft tissue integration of dental implants in vivo [156]. Modifying Ti implants with collagen type I, another cell adhesive protein, has shown positive results in enhancing fibroblast adhesion and proliferation, as shown in Fig. 19.2 [157–159]. One key parameter that needs consideration is the protein conformation, which is known to significantly alter cell adhesion and proliferation especially when a lower concentration of protein is utilized for biomimetic surface modification [160]. Other strategies include modifying surface with chimeric peptides [161], growth factors like platelet-derived growth factor [147], fibronectin [162]. Hydrothermal treatment with calcium and magnesium salts [163] or dompamine coating have been also investigated [164,165]. All these treatments have shown success in enhanced proliferation and differentiation of fibroblasts, [163] and/or epithelial cells [161,163], and in some cases improved soft tissue attachment in vivo [147,161,165].

19.2.3 Modulation of the immune response

The cascade of events following implantation of a material in the body is triggered by the immune system and the fate of the material is dictated by the nature of its interactions

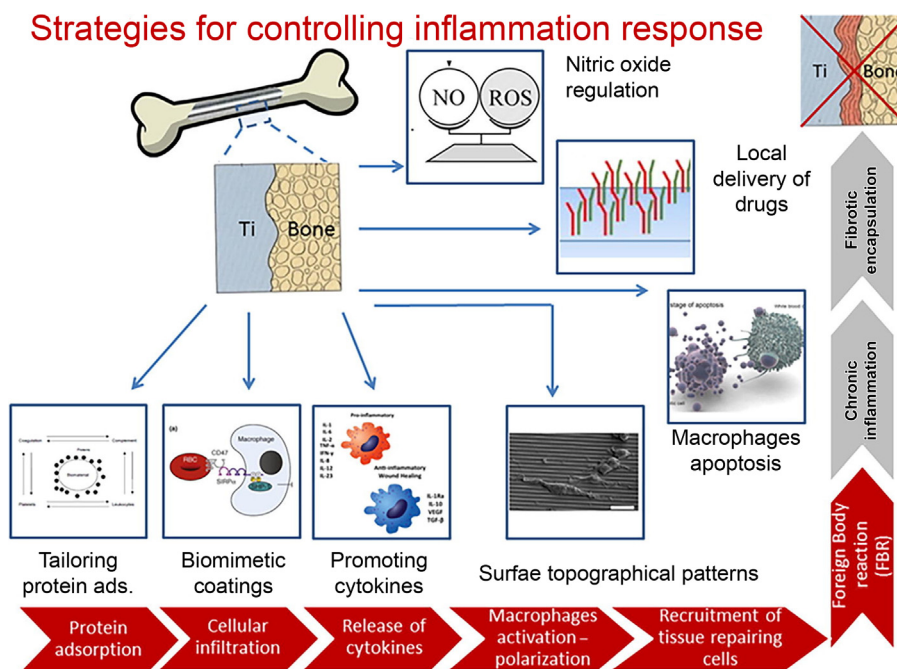


FIGURE 19.3 The inflammatory response pathway and related strategies for controlling inflammation response of Ti implants [173]. Source: Reprinted from Spriano S, et al. A critical review of multifunctional titanium surfaces: new frontiers for improving osseointegration and host response, avoiding bacteria contamination. *Acta Biomater* 2018;79:1–22, with permission from Elsevier.

with the immune cells. The immune cell-material interactions are more relevant in the clinical success of the implant than the interactions of the material with the specific functional cells that eventually will integrate the implant with the surrounding tissue [166–169]. The biological series of events, as highlighted in Fig. 19.3, following the implant introduction into the biological environment include protein adsorption, recruitment of the cells of the innate immune system, migration of neutrophils, substitution by monocytes and differentiation into macrophages, generation of reactive oxygen species, foreign body reaction, and formation of foreign body giant cells via fusion of monocytes/macrophages [170]. Acute inflammation normally lasts for less than a week but in some cases continued activation of the immune system can result in chronic inflammation, which results in destruction of healthy tissue surrounding the implant [67].

Therefore, there has been an increased interest in employing surface engineering-based approaches for modulating the host inflammation response via macrophage polarization, the so-called osteoimmunomodulation [171–173]. Some of these strategies are summarized in Fig. 19.3. During osteomodulation, the bone immune response is modulated by the nature of the implanted material [174]. This relies on the fact that macrophages can either have inhibitory or stimulatory effects on osteogenesis and the nature of effect is surface-mediated. The ultimate goal of this approach is to ensure successful integration of the foreign implant with the surrounding tissue and new tissue formation. Recent studies have

highlighted the critical role played by macrophages. Indeed, the balance between the activation of the cells to M1 (proinflammatory response in case of acute threat or infection) or M2 phenotype (control over wound healing and tissue remodeling) is essential for wound-site healing and implant integration [166,175]. Studies have shown that prolonged M1 polarization and delayed activation of M2 polarization results in chronic inflammation leading to aseptic loosening of the implant [176]. Osseointegration is achieved via timely switch to M2 phenotype which is preceded with secretion of cytokines that promote osteogenesis and tissue remodeling [177,178].

Appropriate tuning of surface properties, especially surface chemistry and topography, has been used by researchers to successfully control osteoimmunomodulation of Ti implants. For instance, increasing surface wettability in combination with nanoscale modifications downregulate M1-associated gene and protein expression supporting the establishment of M2-like macrophage phenotype [179]. Surface roughness and wettability worked in a synergistic manner to create an environment that is conducive to rapid healing and increased osseointegration. Isolated changes in surface wettability can alter macrophage activation and the cytokine release profile [67]. In turn, these differences are achieved by dictating cell shape and plasticity, thereby attenuating inflammation when compared to the untreated controls [180–182]. Surface modification of Ti with nanotubes has been reported to alter macrophage function, that is improved immune cytocompatibility of nanotube-coated surfaces when compared to the untreated counterparts [183–185].

Along with surface topography and surface roughness, surface chemistry can also influence the type of macrophage polarization. Modification of Ti surfaces with 3-aminopropyl triethoxysilane resulted in the least antiinflammatory response and upregulation of M2 polarization via the NF- κ B pathway when compared to titanium surfaces modified using dopamine and sodium hydroxide treatment [186]. Moreover, incorporating boron in calcium silicate coatings deposited on pure titanium promoted M2 macrophage polarization via toll-like receptor signaling pathway [187]. Also, the presence of boron inhibited macrophage differentiation into osteoclast and stimulated osteogenic differentiation of MSCs by activating BMP2 signaling pathway; thereby concluding that incorporation of boron into ceramic coatings could provide beneficial immune responses.

Table 19.1 summarizes surface medication strategies to control osteoimmunomodulation of bone biomaterials (not restricted to Ti but covers broad range of polymeric biomaterials) indicating that these strategies can be applied to Ti-based biomaterials.

19.3 Antimicrobial coatings on titanium

Infection is one of the main causes leading to the failure of orthopedic [196–199] and dental [200–203] Ti implants. Ti surfaces are readily covered by a thin layer of proteins after surgery, which favors bacterial colonization and biofilm formation. Biofilms are a community of multispecies bacteria that are embedded in the matrix of extracellular polymeric substances. The microenvironment of biofilms makes it mechanically stable and more resistant to microbial agents. There is no gold standard to effectively remove established biofilms on biomaterials surfaces and thus, to eliminate the infection. The treatment generally requires a long period of antibiotic therapy and repeated surgical procedures

TABLE 19.1 Surface modification strategies to endow bone biomaterials with favorable osteoimmunomodulation.

Strategies	Methods	Examples	References
Chemical modification	Doping with nutrient elements. Bioactive molecules such as macrophage inducer (IL-4) or inflammatory cytokines (TNF α , IFN γ , oncostatin M (OSM))	Combination of different nutrient elements like Mg, Sr, and Si OSM has gained significant attention for osteoimmunology due to its ability for regulating osteogenesis and osteoclastogenesis	[188–190]
Fabrication method	By altering the material size from macro to micro to nano By controlling the pore size and porosity of 3D printed scaffolds	Inert materials can become immunogenic when in nano-size Uniform micron size pores (30–40 μ m) supports polarization toward M2 phenotype whereas nanoporous or random porous-sized materials favor M1 phenotype	[191–193]
Surface modification	Modifying surface topology (size and roughness) and surface chemistry (wettability and surface charge)	Nanoscale structures or surfaces with different hydrophilicity alter protein expression profiles and cytokine release	[183,194]
Others	Using immunomodulatory drugs	Local administration of antiinflammatory drugs enhance bone regeneration outcome by regulating the immune response toward implanted material	[195]

With advancement in surface engineering approaches, efforts are being made to design a multifunctional surface which is not only bioactive but also antibacterial and possess immunomodulatory properties. Such properties will ensure successful integration of the implanted material with the surrounding tissue evading chronic inflammation and infection-related problems. However, limited success has been achieved in designing a bioactive and antibacterial surface that can modulate inflammation due to uncontrolled inflammation, which can trigger progression of osseointegration to chronic inflammation. Despite significant success achieved in preparing bioactive and antibacterial surfaces, optimization of surface properties of implants is still required to conceive a surface that is capable of modulating inflammation but also stimulating physiological osseointegration so that infection and chronic inflammation are avoided.

Adapted from Chen Z, Klein T, Murray RZ, Crawford R, Chang J, Wu C, et al. Osteoimmunomodulation for the development of advanced bone biomaterials. Mater Today 2016;19(6):304–21.

[204]. Antimicrobial coatings might be an efficient technological approach to prevent, instead of treat, bacterial colonization and reduce infections. Numerous solutions have been proposed, including coatings made of antibiotics, antimicrobial peptides (AMPs), and other organic and inorganic antimicrobial agents [205,206]. The representative coating strategies employed are listed in Table 19.2. The antimicrobial activity of these solutions has been demonstrated either in vitro or in vivo, but the biocompatibility of these coatings also needs to be considered before translation for clinical use.

19.3.1 Coatings

19.3.1.1 Antibiotic coatings

Systemic antibiotics are typical postsurgical antimicrobials given to patients. However, biofilm-embedded pathogenic microbes are less susceptible to antibiotics. Immobilizing

TABLE 19.2 Representative antimicrobial coatings on titanium implants antimicrobial agent.

	Coating method	Test condition	Bacterial strain	Results	Reference
Antibiotic					
Gentamicin	Loaded in poly(D, L-Lactide)	In vivo (rat and patient)	<i>Staphylococcus epidermidis</i>	80%–90% infection prevention in rats, none infection in 8 patients for 1 year	[207]
Gentamicin	Immobilized on hydroxyapatite	In vivo (rabbit)	<i>Staphylococcus aureus</i>	No infection, induce new bone formation	[208]
Gentamicin	Immobilized on heparinized-Ti	In vitro	<i>S. aureus</i>	Inhibit bacterial growth for 10 days, no cytotoxicity with osteoblast cells	[209]
Vancomycin	Covalent binding	In vitro	<i>S. aureus</i>	91% decrease in colonization, no biocompatibility test	[210]
Vancomycin	Sol–gel film	In vitro, in vivo (rat)	<i>S. aureus</i>	Minimal sign of infection, inhibit bone resorption	[211]
Vancomycin	Covalent binding	In vitro	<i>S. epidermidis</i> , <i>Escherichia coli</i>	Specifically inhibit <i>S. epidermidis</i> but not <i>E. coli</i> , no biocompatibility test	[212]
Tobramycin	Immobilized on hydroxyapatite	In vitro	<i>S. aureus</i>	Inhibit bacterial growth, no biocompatibility test	[214]
Antimicrobial peptide					
Tet213 (KRWWKWWRR)	Immobilized on hydroxyapatite	In vitro	<i>S. aureus</i> , <i>Pseudomonas aeruginosa</i>	Kill both strains within 30 min, no cytotoxicity with osteoblast-like cells	[219]
HHC-36 (KRWWKWWRR-NH ₂)	Immobilized on calcium phosphate-phospholipid multilayer	In vitro	<i>S. aureus</i> , <i>P. aeruginosa</i>	Eradicate biofilm growth, no cytotoxicity with osteoblast-like cells	[220]
GL13K (GKIIKLKASLKLL-NH ₂)	Covalently binding	In vitro	<i>Porphyromonas gingivalis</i> , <i>Streptococcus gordonii</i>	Inhibit biofilm growth, no cytotoxicity with fibroblast and osteoblast cells	[221,222]

LKLLKKLLKLLKKL	Linked as chimeric peptide with solid binding peptide	In vitro	<i>Streptococcus mutans</i> , <i>S. epidermidis</i> , <i>E. coli</i>	Significantly reduce bacteria adhesion, no biocompatibility test	[226,227]
Melimine (CTLISWIKNKRKQRPVSRRRRRRGRRRR)	Covalently binding	In vitro, in vivo (mouse and rat)	<i>S. aureus</i> , <i>P. aeruginosa</i>	Reduce in vitro bacterial adhesion and bacterial load in animal infection model	[223]
LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES)	Covalent binding	In vitro	<i>E. coli</i>	Kill bacteria on contact, no biocompatibility test	[224]
hLf1–11 (GRRRRSVQWCA-NH ₂)	Covalent binding	In vitro	<i>Streptococcus sanguinis</i> , <i>Lactobacillus salivarius</i>	Inhibit bacterial growth, no cytotoxicity with fibroblast cells	[225]
Other organic antimicrobials					
Chlorhexidine	Immobilized on hydroxyapatite	In vivo (goat)	<i>S. aureus</i>	Decrease infection, improve fixation of external fixator pints	[228]
Chlorhexidine, chloroxylenol	Dipping	In vitro	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>P. aeruginosa</i> , <i>E. coli</i> , <i>Candida albicans</i>	Inhibit bacterial growth for up to 8 weeks, no biocompatibility test	[245]
Chitosan	Covalent binding	In vitro	<i>Actinomyces naeslundii</i> , <i>P. gingivalis</i>	Inhibit <i>A. naeslundii</i> but not <i>P. gingivalis</i> no cytotoxicity with fibroblast cells	[229]
Poly (amidoamine) dendrimers	Immobilized on calcium phosphate	In vitro	<i>S. aureus</i> , <i>P. aeruginosa</i>	Inhibit bacterial colonization, low cytotoxicity with human bone mesenchymal stem cells	[230]
Inorganic antimicrobials					
Silver	Immobilized on titania nanotubes	In vitro	<i>S. aureus</i>	Prevent bacterial adhesion for 30 days, some cytotoxicity with osteoblast cells	[233]
Silver	Immobilized on hydroxyapatite	In vitro	<i>Scaphirhynchus albus</i> , <i>E. coli</i>	Kill more than 90% bacteria, good biocompatibility with osteoblast cells	[234]

(Continued)

TABLE 19.2 (Continued)

	Coating method	Test condition	Bacterial strain	Results	Reference
Silver	Plasma immersion ion implantation	In vitro, in vivo (rat)	<i>S. epidermidis</i>	Reduce biofilm and no cytotoxicity in vitro, reduce bacterial infection in vivo	[235]
Iodine	Anodic oxidation	In vitro, in vivo (rabbit)	<i>S. aureus</i> , <i>E. coli</i>	Inhibit bacterial colonization, no cytotoxicity with fibroblast cells, fewer signs of infection in vivo	[237]
Iodine	Anodic oxidation	In vivo (patient)	N/A	Prevent postoperative infections with no detection of cytotoxic or adverse effects	[238]
Nanostructures					
Nanorough	Electron beam evaporation	In vitro	<i>S. aureus</i> , <i>S. epidermidis</i> , <i>P. aeruginosa</i>	Decrease bacteria adhesion, no biocompatibility test	[242]
80 nm TiO ₂ nanotubes	Electrochemical anodization	In vitro	<i>S. aureus</i> , <i>S. epidermidis</i>	Decrease bacteria adhesion, no biocompatibility test	[244]

antibiotics onto implant surfaces provides a local effect at the targeted site and localization of a relatively high drug concentration. Gentamicin and vancomycin are the two mostly studied antibiotics applied on Ti implants [207–212]. Other tested antibiotics include tobramycin, amoxicillin, cefamandol, carbenicillin, and cephalothin [213,214]. The antibiotic coating can significantly reduce or inhibit the in vitro colonization of implant-associated pathogens, including *Staphylococcus aureus* and *Staphylococcus epidermidis*. Furthermore, these antibiotic-coated Ti implants have shown effectiveness in reducing or eradicating infections in in vivo tests (e.g., rats, rabbits, and patients). Since these antibiotics are widely used as systemic antibiotics after surgeries, their coatings generally present good biocompatibility in vitro and in vivo. Before antibiotic-coated Ti implants are applied to patients, some problems still need to be addressed, such as the drug resistance of bacteria, long period of drug delivery, optimal released concentration, and possible cytotoxicity [215].

19.3.1.2 Antimicrobial peptide coatings

With the developing of conventional antibiotic resistance, alternative antimicrobial agents are constantly studied [216]. Given the intrinsic properties of broad spectrum activity and rapid acting, AMPs are considered as a promising next generation of antibiotics [217]. Based on the bactericidal mechanism of rupturing bacterial membrane by contacting, immobilized AMPs preserved the antimicrobial activity on a variety of biomaterial surfaces, such as resin, contact lenses, and Ti [218]. Immobilization of AMPs on Ti surfaces could be performed by incorporating in the coating of calcium phosphates [219,220], by direct covalent binding on Ti, [221–225] or used in combination with solid binding peptides as chimeric peptides [226,227]. AMPs used for modifying Ti surfaces (e.g., Tet213, HHC-36, GL13K, Melimine, LL37, and hLf1–11) are short molecules of less than 50 amino acids with cationic and hydrophobic residues. AMP coatings on Ti have shown antimicrobial activity in vitro against Gram-positive *S. aureus*, *Streptococcus gordonii*, *Streptococcus sanguinis*, and *Lactobacillus salivarius* as well as Gram-negative *Pseudomonas aeruginosa*, *Porphyromonas gingivalis*, and *Escherichia coli* [221–225]. Fig. 19.4 shows an example of killing *S. gordonii* on GL13K peptide-coated Ti surfaces. No significant cytotoxicity has been found with fibroblast and osteoblast cells on AMP-coated Ti. The biocompatibility of Melimine has also been tested in mouse and rat models [223]. Substantially more work needs to be done with AMP coatings to better understand their bactericidal mechanisms, reduce potential cytotoxicity, and produce more robust (resistant to proteinases and other degradative agents) and cost-effective coated implants.

19.3.1.3 Other organic antimicrobial coatings

Other than antibiotics and AMPs, researchers have also applied many different organic coatings onto Ti surfaces. Antiseptics are good alternatives for their properties of low cytotoxicity, broad spectrum activity, and low bacterial resistance. For example, chlorhexidine is widely used in dental settings like periodontal surgery or mouth wash and has been used to coat Ti implant surfaces with good performance in reducing postsurgical infection in goats [228]. Chlorhexidine has also been used in a coating with another antiseptic, chloroxylenol, and showed good antimicrobial activity against many types of microbes, including *S. epidermidis*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *Candida albicans* [229]. Other examples

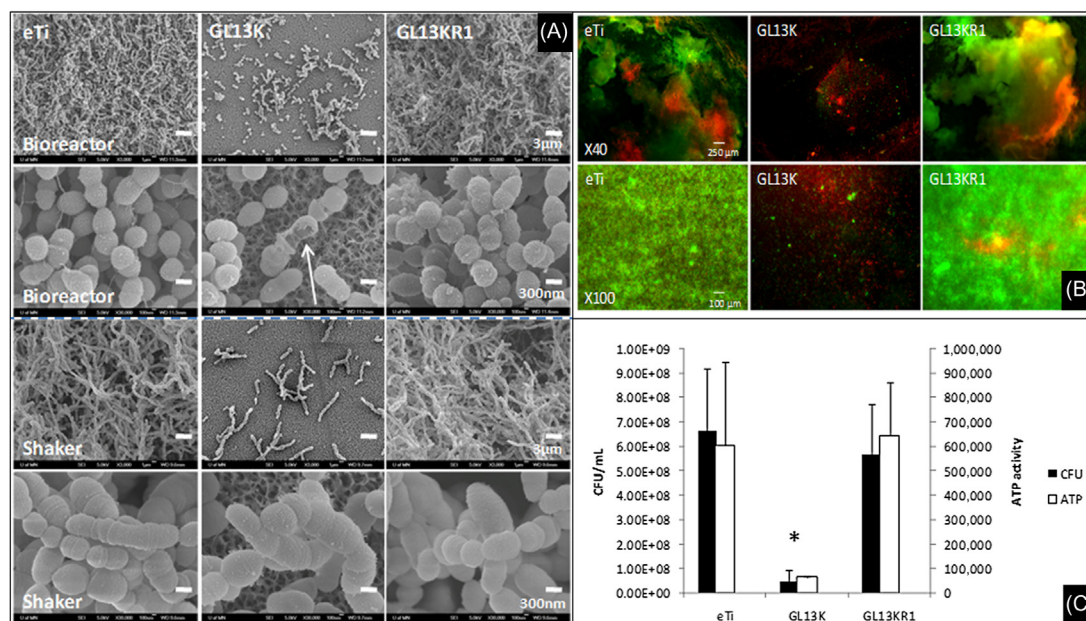


FIGURE 19.4 (A) FE-SEM images of *Streptococcus gordonii* biofilms grown on different titanium surfaces tested in the drip flow bioreactor and orbital shaker. (B) Live/dead cell imaging of *S. gordonii* cultured on GL13K coated surfaces for 3d in the drip flow bioreactor. (C) Colony forming unit (CFU) and adenosine triphosphate (ATP) activity of *S. gordonii* bacteria cultured on differently treated surfaces [221]. Source: Reproduced from Chen X, Hirt H, Li Y, Gorr SU, Aparicio C. Antimicrobial GL13K peptide coatings killed and ruptured the wall of *Streptococcus gordonii* and prevented formation and growth of biofilms. *PLoS One* 2014;9(11):e111579. <<https://doi.org/10.1371/journal.pone.0111579>>, <<https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0111579>>.

of biomolecules immobilized on Ti surfaces include chitosan [229] and poly(amidoamine) dendrimers [230].

19.3.1.4 Inorganic antimicrobial coatings

Organic antimicrobial coatings are generally susceptible to biodegradation by enzymes or bacteria in the human body. In comparison, inorganic coatings are more stable and have less risk of losing antimicrobial activity after long periods of implantation [231]. Silver ions or silver nanoparticles have been long and widely used as broad spectrum bactericidal agents [232]. Silver has been immobilized on Ti surfaces by electrostatic interaction with another layer of titania nanotubes [233] or HA [234]. The coating of silver on titania nanotubes showed efficient inhibition of bacterial colonization for up to 30 days in vitro, but also presented cytotoxicity effects on osteoblast cells [233]. The cytotoxicity can be reduced by loading less silver in the coatings or by incorporation of the ion using other coating methods [233–235]. Considering the possible cytotoxicity of silver, other inorganic antimicrobial agents are available to be applied in the coating. Iodine-based antiseptics, such as povidone iodine, provide broad spectrum activity, low cytotoxicity, and

good tolerability [236]. Furthermore, iodine is one of the essential trace elements in human health, which suggests good biocompatibility. Applying iodine coatings on titanium implant surfaces reduced bacterial colonization in vitro and prevented infections in animal models and clinical trials [237,238]. In addition, inorganic coatings can be combined with organic antimicrobial agents as hybrid layers, such as silver nanoparticles with chitosan [239]. These inorganic–organic hybrid antimicrobial coatings will be further explored as they can combine properties and/or used to retain activity and simultaneously overcome drawbacks of each component of the components.

19.3.2 Surface nanostructures to prevent bacteria colonization

Nanotexturing is generally explored to encourage the cellular attachment and proliferation, but usually comes with the problem of more bacterial retention due to increased surface area [240]. However, surface modification with nanostructures of certain geometries and sizes can prevent bacterial adhesion, which is related to the reduced adhesion energy between the nanostructure and bacteria [241]. This property has also been applied to Ti surfaces. For example, among a series of different nanotextured Ti surfaces only nanorough Ti surface fabricated by electron beam evaporation decreased bacterial adhesion [242]. TiO₂ nanotubes synthesized by electrochemical anodization usually attract more bacteria [243], but Ti nanotubes with 80 nm diameter can effectively reduce bacterial adhesion relative to smooth Ti surface or Ti nanotubes with smaller or larger diameter [244].

19.4 Conclusion

Titanium and its alloys have been used as implantable materials for replacing bone and teeth for several decades. Different Ti surface modification strategies have been highlighted in this chapter to improve cellular interactions with Ti surfaces and thus, improve clinical outcomes. The surface modification of Ti implants can be done by applying a single technology, but recently the combination of a number of them has been pursued so that a multifunctional surface is obtained. An ideal multifunctional Ti surface would integrate active cues and/or agents to (1) induce repair and regeneration of soft and hard peri-implant tissues, (2) induce favorable immunomodulation response, and (3) prevent bacterial colonization and/or biofilm formation. Some of the surface modification techniques mentioned here have been tested in clinical settings but continuous advances still need to be made to ensure more robust and reproducible Ti surfaces that better tune cellular responses. Careful understanding of the complex host–implant interaction and the clinical site (dental, orthopedic, etc.) will be critical for designing future strategies for modifying Ti implant surfaces that will be more effective for tissue repair and regeneration.

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Cellular response to metal implants

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20.1 Introduction

A broad range of materials including metals, ceramics, polymers, and their composites are used for fabrication of implants. Metals and alloys have been employed for biomedical implant applications over the last 100 years, since they have shown some advantages as compared to other materials, such as high mechanical properties, ductility, good conductivity of electricity and heat, and chemical/biological compatibility. “Lane plate,” an internal fixation plate used for bone fractures, was the first metal implant outside of dental implants, that was introduced in 1895 [1]. Development of modern metal implants was initiated with the development of stainless steel and cobalt–chromium alloys in the early 1900s. Since then, metal implants including orthopedic, dental, and cardiac devices were introduced in the middle of the 20th century [2,3]. The first total hip replacement (THR) containing metal-on-metal (MoM) designs with cobalt–chromium alloys was developed in the 1950s and 1960s [4]. In the following years with more advances in technology, metal implants branch into almost all medical fields in order to treat and control a wide variety of diseases. At present, there are numerous types of metal implants due to progress in development in the recent years.

All biomaterials implanted in the body can potentially produce a host inflammatory response. Acute inflammatory response, initiated by injury to the tissue, is one of the factors defining the biological response to implants in short and long periods of time. The type and location of a surgical procedure, and the biological makeup of the host influence the severity of the acute inflammatory response. The implant, its composition, its physicochemical properties, its relationship to the host in addition to its degradability and production of ions, and other byproducts are other determining factors on the biological response to the implant [5,6].

Designing of modern implants is conducted to stimulate an appropriate cellular response to develop implant integration while inhibiting from its perpetuation, results in chronic inflammation and foreign body reactions, and finally reduction in implant function [7].

This chapter primarily presents brief information about presently used metals and their common uses in the medical device implants and then summarizes the general knowledge of immune responses to implants and outline the immunomodulation of biomaterials to induce specific biological responses.

20.2 Metallic implants

Metals that have been successfully used in orthopedic applications include stainless steel, titanium and its alloys, and cobalt–chromium-based alloys. Titanium and its alloys became materials of great interest in biomedical applications due to their excellent properties such as corrosion resistance, low density, biocompatibility and lower modulus. Formation of a hard TiO_2 film at the surface of Ti and its alloys, results in superior corrosion resistance and therefore a high level of biocompatibility. Ti and Ti alloys represent firmly integration with bone tissue due to easy bonding with bone, leading to longevity and reduction in the failure risk of the implant [8,9]. The most common Ti materials used in implants are commercially pure Ti and Ti–6Al–4V alloy. However, release of Al and V in long-term applications can potentially cause Alzheimer's, osteomalacia, and neuropathy, which led to the development of Ti–6Al–7Nb and Ti–5Al–2.5Fe, the vanadium-free alloys, and TNZT alloys based on Ti, Nb, Ta, and Zr [9,10]. Fretting and poor wear resistance of Ti alloys can induce allergic tissue rather than other alloys and also limit their applications. Wear resistance of Ti alloys can be improved through surface treatment techniques including TiN coating, thermal oxidation, and ion implantation [8,10].

Cobalt–chromium alloys used in biomedical applications include Co–Cr–Mo alloy (cast) and Co–Ni–Cr–Mo alloy (wrought). Cobalt–chromium-based alloys display high corrosion resistance due to formation of a passive oxide film. They also have good wear resistance, fatigue resistance, and high elastic modulus similar to that of stainless steel and more than that of cortical bone. Since cobalt has lower reactivity than titanium, the oxide film is not quickly formed like titanium, leading to small degrees of corrosion. Therefore, release of metal ions such as Ni, Cr, and Co that have been considered toxic ions, can cause an allergic reaction. Thermal treatment of cobalt–chromium alloys can change the mechanical and electrochemical properties of these implants [9,10].

Stainless steel materials due to high Cr content, are resistant against a wide range of corrosive agents. High affinity of Cr in stainless steel for oxygen, results in formation of a hard and corrosion resistant coating of Cr_2O_3 . Among the available types of stainless steel, austenitic stainless steel is the most used type for the implant fabrication. The stainless steel that is widely used in traumatological temporary devices, is 316L containing Co, Cr, Ni, and Mo. Austenitic stainless steel has rather low wear resistance. Therefore, the production of a high amount of wear debris results in fast loosening which limits their application in orthopedic joint prosthesis such as the hip [9,10].

20.2.1 Orthopedic devices

A wide range of medical implants belong to orthopedic implants that present specific biological functions via substituting a missing joint or repairing any damaged tissues such as bone, cartilage, or ligaments and tendons [9]. Orthopedic implants are mainly based on cobalt, chromium, molybdenum, nickel, titanium, and zirconium alloys. Orthopedic implants include THR, total knee replacement (TKR), spinal disk replacement, shoulder replacement, bone screw, and bone fixation devices such as nails [8].

Hip joint implants consist of femoral stem, femoral head, and acetabular component. Metals currently used in hip joints are titanium-based alloys, Co–Cr alloys, and 316L stainless steel. The femoral stem that is subjected to the highest mechanical stresses is firmly inserted into the femoral bone. The metallic or ceramic femoral head is fit on the end of the stem via a taper junction. The acetabular liner represents the tribological surface worn by the action of the femoral head. The acetabular cup, fits into the pelvis, either by bone cement or by press-fitting. Acetabular cup is mainly made of ultrahigh molecular weight polyethylene (UHMWPE) [10–12].

TKR commonly is made of three parts: femur, tibia, and patella or kneecap. The femoral part and tibia are metallic components manufactured from titanium alloy, stainless steel, or cobalt–chromium with a low amount of molybdenum. The femoral part attaches to the end of the resurfaced femur (tightbone). Tibia is the flat component, attaching to the top of the resurfaced shinbone at the tibia. Patella is the dome-shaped piece replacing the damaged kneecap that rubs against the tightbone. The tibia insert or spacer and the patella components are typically manufactured from plastics such as UHMWPE [10,13]. Steel implants for knee replacement, due to higher stiffness of steel rather than bone, are generally fixed with bone cement in order to reduce the stress shielding effects [14]. Due to release of iron from stainless steel alloys in the body and then bacterial infections, these alloys are used for short-term or as coated implants (to reduce the negative effects). Titanium-and cobalt-based alloys were recently used for fabrication of knee implants [8].

Bone screws, used to fixing elements in prosthetics, are commonly made of steel and titanium but magnesium and magnesium-coated screws have been recently developed for improved performance [8].

20.2.2 Cardiac and endovascular implants

Coronary stents are expandable tubes of metallic mesh used to prevent vascular recoil and then preserve the patency of the vascular lumen [15]. Classification of stents are according to the mechanism of expansion (self-expanding or balloon expandable), composition (stainless steel, cobalt-based alloy, tantalum, nitinol (nickel–titanium), inert coating, gold coating, active coating, no coating or biodegradable), and design (mesh structure, coil, etc.) [15]. Stainless steel 316L and cobalt–chromium alloy F562 or L605 with a coat of gold or nitinol alloy are usually used in manufacturing of coronary artery stents [16].

Pacemakers and implantable cardioverter defibrillators (ICDs) are devices for treatment of irregular heart rhythms. Pacemakers are implanted to treat slow heart rhythms, called bradycardia while ICDs detect dangerous fast heart rhythms and are able to deliver a small electrical shock via wires connected to heart to stop them. New generation of ICDs

also can function as pacemakers. Pacemakers and ICDs are composed of pulse generator and lead. Pulse generator is often covered with a titanium capsule and alloy leads are insulated with polymer or silicon. Leads connect to the capsule through the header of the pacemaker and deliver the impulses to the heart to electrically stimulate the heart via the pacing electrodes. Leads also relay the heart information back to the pulse generator. The conductor wires are made of MP35N (an alloy of Ni, Co, Cr, and Mo) or may contain a silver core for high-voltage applications (principally automatic implantable cardioverter defibrillation). The pacing electrodes are manufactured from platinum-iridium alloy. ICD leads also have the same pacing electrodes at the tip in addition to one or two defibrillation electrodes (shock coils) in order to deliver high-energy cardioversion pulses [15,16].

Patent foramen ovale (PFO) occluders, a self-expanding double disk device, are made from nitinol wire. PFO devices are used to repair the atrial septal defects in the walls of heart chambers [17].

20.2.3 Dental and oral/maxillofacial devices

Nowadays, metallic biomaterials are widely used in dental applications. The most common type of dental implants is endosseous dental implants which are used to achieve osseointegration of the bone with the implant and are supported by dental restorations such as crowns and bridges [18,19]. Endosseous implants are embedded in the maxilla or mandible via an intraoral incision in the mucoperiosteum in order to replace the root. Commercially pure titanium or titanium alloy such as Ti-6Al-4V is used for endosseous implants [19,20].

Dental appliances such as orthodontic wires are used to carry out the tooth movement. Ti and Ni are used in dental appliances and dental restorations. Stainless steel, titanium-nickel, and cobalt-chromium alloys are used for manufacturing orthodontic arc wires [21,22]. Metal alloys such as cobalt-chromium and nickel-chromium are used to fabricate crowns and bridges [23]. Titanium or titanium alloys are also used in manufacturing of metal plates and screws in order to obtain fixation of the bone in the oral maxillofacial areas and fracture healing [21,24].

Temporomandibular joint (TMJ) implants, complex devices that are used to obtain a biomechanical solution for reconstruction of TMJ, are made from the cobalt-chromium alloy [25]. Dental amalgam, an alloy used in dental restoration applications, is composed of a mixture of liquid mercury (Hg) and a powder consisting of silver, tin, copper, and other trace metals containing zinc [26].

20.2.4 Neurological devices

Flow diverters are stent like devices for the endovascular treatment of intracranial aneurysms. These devices are placed on the aneurysm neck along the parent artery to divert flow away from the aneurysm [27,28]. Pipeline embolization device (PED) and the silk flow diverter (SFD) are two commercially available flow diverters. PED, a mesh tube woven wire, is composed of 75% cobalt-nickel alloy and 25% platinum. SFD, the flexible and self-expanding device is composed of 48% nitinol and platinum microfilaments [29].

Stent-assisted coiling for embolization is placed across the aneurysm neck to obstruct blood flow into the sac and support the artery. The goal of this device is to exclude the aneurysm from the circulation [28]. Aneurysm clips are used to keep the aneurysm's neck closed preventing arteries from rupturing and isolating the ruptured aneurysms from the intracranial circulation [30]. Generally, intracranial devices for aneurysm treatments, containing aneurysm clips, flow diverters, and endosaccular occlusion devices, are manufactured from nickel and its alloys with other metals [31].

20.2.5 Gynecological devices

Metals used in gynecological devices are copper, titanium, and nickel. Intrauterine devices (IUD), consisting of copper (Cu), are used to induce reversible contraception. IUDs with spermicidal function inhibit the production of viable embryos through changing in endometrium [32]. Copper IUDs such as Paragard 380A, are considered as relatively pure, consisting of 99.9% pure copper, polyethylene, and barium sulfate [33]. Release of Cu ions from copper IUDs after insertion induces a local inflammatory response that is often limited to the uterus cavity and genital tract leading to contraceptive effects [32].

Permanent contraceptive devices, such as Essure hysteroscopic tubal occlusion device, are implanted transvaginally in Fallopian tubes inducing an inflammatory response that leads to fibrosis and tubal occlusion. This device is designed to change the function of Fallopian tubes resulting in permanent contraception. Essure is based on nitinol alloy with 55% titanium and 45% nickel in outer coils and 316L stainless steel in inner coil [33,34].

20.3 Corrosion and metal ion release

Metallic corrosion, described as metal degradation, is an electrochemical process (oxidation and reduction reactions) that depends on the environment, pH values, and electromotive potential. Corrosion can take place on all metal surfaces. Release of metal ions at implant surfaces in the body due to electrochemical reactions results in implant degradation. An electromotive potential lower than a certain value will cause immunity from corrosion and at higher values, the pH level defines whether the metal will corrode or form a layer on its surface. This layer resulted from a chemical reaction with the environment. Nowadays, corrosion of orthopedic implants usually is too low to be obvious. Obvious corrosion typically takes place in cracks, at parts junction, or due to stress and micro motion. Five common forms of corrosion could take place after biomaterial implantation: general, crevice, pitting, fretting, and galvanic [35].

In general corrosion, metal ions are released uniformly from the surface. The amount of released ions from implant with surface oxides, depends on the structure and composition of oxide layer [36]. Upon implantation, the amount of metal ion release usually is the highest, and the release rate decreases over time. Where the oxide layer is not protective, metal ion release lasts longer and the rate of the release increases after implantation.

In pitting corrosion, there are pits or cavities on the surface of metal implant, penetrating the implant surface during that time. Pits that are usually round or cup shaped,

develop in cracks or surface defects. These cavities lead to metal ion release or metal dissolution in the media and in worse conditions could hazard the integrity and function of the biomaterial [37].

Fretting corrosion is the result of micro motion between assembled parts and contacting surfaces of a device. Wear debris produced by this motion, because of higher surface area per unit volume, are more susceptible to corrosion than the bulk material and cause additional corrosion. This motion disrupts any passive oxide film leading to increased wear and then release of a noticeable amount of toxic material. Therefore, inhibition from micro motion in implant assemblies should be paid attention to. This form of corrosion has been reported in implants with modular designs such as hip and knee replacement devices [38,39].

Galvanic corrosion occurs when two metals with different electromotive potential are in contact in a conductive environment. An electromotive force is then produced by the metals contact. Corrosion occurs at the anode, while the rate of corrosion in cathode is slower than it would be alone. The rate of corrosion depends on the voltage difference and the ratio of surface area between the different metals [39].

A crevice corrosion usually occurs in contact areas between the implant and a small volume of stagnant liquid. The chemistry of the media can be different in the crevices leading to oxygen depletion and reduction in pH which cause more susceptibility of surface to corrosion [39].

20.4 Cellular response to metal implants

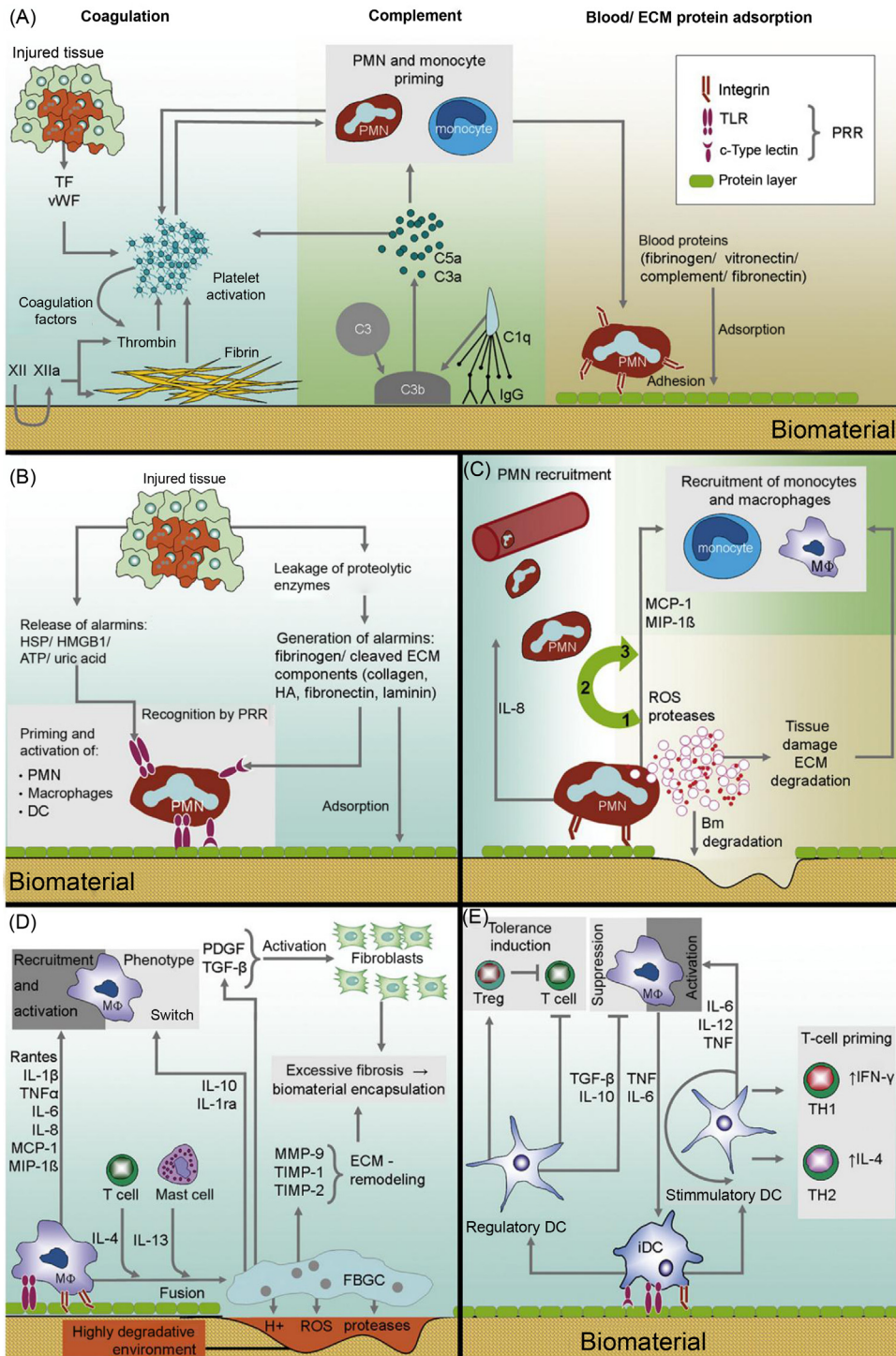
The initial injury to the tissue surrounding the implant after implantation induces an acute inflammatory reaction, activating the innate immune response. Several factors such as composition, structural form, and the amount of exposed metal of the implant in addition to tissue physiology and immunology, may affect the severity of the innate responses, inflammatory responses, and the success or failure of metallic implants [40,41].

20.4.1 Inflammatory response

Just after the implant is placed and direct contact of the implant is made with the tissue, proteins from blood (or serum) rapidly adsorb to the implant surface before interaction with cells. Properties of adsorbed protein including the type and concentration, depends on the physicochemical properties of the implant [42]. This formed protein layer characterizes the tissue response to the biomaterial and conducts the activation of complement and coagulation which form a matrix around the biomaterial resulting in initiating the inflammatory response [40,43,44] (Fig. 20.1A).

20.4.1.1 Coagulation, complement activation, and protein adsorption

Factor XII (FXII), the initiator of the intrinsic pathway of coagulation, is contact activated on negatively-charged surfaces and in following, a cascade of protein reactions leads to production of thrombin [43,46]. Since the amount of produced thrombin by activation of FXII is not enough for blood coagulation, the mix of both contact activation and platelet activation is needed [47,48]. Even low amounts of thrombin make the platelets active to



release mediators required for coagulation, creating the phospholipid surface for the coagulation [47,49]. Attached fibrinogen/fibrin to implants can activate integrin bonding domains on phagocytes and therefore cause more inflammatory response and coagulation [50,51]. In addition to thrombin and fibrinogen, the expression of the extrinsic coagulation cascade initiator, and the tissue factor, can activate platelets adherent to the implant [52].

Three separate pathways including the alternative, the classical, and the lectin, initiate complement activation upon contact with the implant. All the pathways converge on the C3 convertase activation level, mediating the production of the anaphylatoxins C3 and C5a [53]. Activation of complement is always related to the protein layer adsorbed on the implant surface. The assembly of the initial enzyme of the classical pathway, C1, via binding of the attached IgG to C1q, can foster the initiation of classical C3 convertase [54]. Moreover, the assembly of the initiating C3 convertase of the alternative pathway is fostered by the adsorbed C3 on implant surface [55]. Due to the function of C3 convertase, C3b which binds to the protein layer of the biomaterial is produced and generated further C3 convertase [55,56]. Upon the start of complement cascade, large amounts of C3a and C5a are released at the implant site [57]. The functions of these both anaphylatoxins containing vascular permeability enhancement, activation of monocytes and granulocytes, and stimulation of granulocyte ROS (reactive oxygen species) release, can induce the inflammatory responses [53].

As described above, the extracellular matrix (ECM) proteins such as fibronectin and vitronectin adsorb on the surface of biomaterials [58]. Fibrinogen and complement activate the inflammatory cells, while fibronectin and vitronectin regulate the inflammatory response [59,60]. Furthermore, these two proteins play a significant role for integration of orthopedic devices due to influence on attachment and spreading of osteoblast cells to implants [61]. The adsorbed protein layer has a determinative effect on cell attachment and activation on the implant surface, through the interaction of proteins with adhesion receptors which results in the following wound-healing response [62,63].

20.4.1.2 Danger signals and recognition

One of the multiple inflammatory pathways started by recognition and uptake of metal debris is pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectins. Pathogen-associated molecular patterns (PAMPs), are detected by PRR, promoting inflammation response [64–66]. Alarmins or danger-associated molecular pattern, endogens equivalent of PAMPs, are immediately released by injured host tissues that contain heat-shock proteins, heparin sulfate, and biglycan fragments [67–69]. The danger signals that include ECM components adhere to implants surface and form a layer (Fig. 20.1A). The resulted implant-associated alarmins can be recognized by innate immune cells such as macrophages via PRRs, especially TLRs on leukocytes and therefore cause an inflammatory response [70,71] (Fig. 20.1B).

FIGURE 20.1 Immune response to biomaterials. (A) Adsorption of blood proteins, coagulation, and complement activation. (B) Release of danger signals from injured tissue. (C) The acute inflammatory response dominated by PMNs function. (D) Chronic inflammation induced by macrophages as driving force. (E) Activation of DCs on the biomaterial surface by macrophage-derived cytokines and PRR engagement. DCs, Dendritic cells; PMNs, polymorphonuclear leukocytes; PRRs, pattern recognition receptors. Source: Reprinted from Franz S, Rammelt S, Scharnweber D, Simon JC. Immune responses to implants—a review of the implications for the design of immunomodulatory biomaterials. *Biomaterials* 2011;32(28):6692–709 [45] with the permission from Elsevier.

20.4.1.3 Activation of inflammatory cells

Polymorphonuclear leukocytes (PMNs) rapidly move from blood to biomaterial site after injury to provide the first line of host defense. Activated platelets and endothelial cells in addition to damaged cells, secrete chemoattractants to employ PMNs at the infection site (Fig. 20.1A and B). On the other hand, degranulation of the mast cells and release of histamine also guide PMNs and monocytes to the implantation site [72,73]. At the biomaterial site, PRRs and integrins are engaged on PMNs surface, inducing phagocytic response [74] (Fig. 20.1C). In order to eliminate pathogens, PMNs release proteolytic enzymes and ROS [75]. Upon activation, PMNs produce immunoregulatory signals including interleukin-8 (IL-8) [76–78], monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 β (MIP-1 β), the chemokines that are secreted from PMNs, direct monocytes, macrophages, immature dendritic cells (DCs) and lymphocytes [75,79]. With ending the phagocytosis function of PMNs and the absence of more activation signals, they endure apoptosis and are eliminated by macrophages. Macrophage phenotype can be changed to an antiinflammatory type due to phagocytosis of apoptotic PMNs, resulting in limitation of inflammatory reactions. Usually 2 days after implantation, there are not any PMNs at the biomaterial site [80,81].

20.4.2 Chronic inflammation

Further production of wear debris can induce chronic inflammation, leading to the release of multiple cytokines by macrophages and foreign body giant cells (FBGCs). Monocytes migrate from the bloodstream to the biomaterial site and differentiate to macrophages. Attachment of macrophages to the implant and the release of chemokines such as IL-8, MCP-1, MIP-1 β can activate more inflammatory cells [82] (Fig. 20.1D). The various functions of macrophages are referred to as M1 (classically activated) and M2 (alternatively activated) macrophages [83,84]. These subsets were then recategorized on the basis of macrophage functions related to the maintenance of hemostasis [85] (Fig. 20.2). Response to the endogenous stimuli secreted from damaged cells and innate immune cells or to adaptive immune signals result in a generation of different macrophages [86,87]. Classically activated macrophages release inflammatory cytokines and produce ROS and nitrogen radicals for microbicidal activity which makes them play a significant role in host defense. Classically activated macrophages are induced by interferon- γ secreted from natural killer (NK) cells or T helper 1 cells or by other activator containing TNF α [85,86]. Macrophages with high phagocytic potential, display classically activated phenotypes [88]. Phagocytosis of large particles requires the fusion of macrophages into FBGCs. Large sized particles and metal debris more than 10 μm in diameter are surrounded by macrophages and FBGCs [89]. FBGC surrounding large metal debris has been reported in histological tissue sections from patients with failed implants [69,81].

Wound-healing macrophages with phagocytosis of debris, secretion of enzymes, cytokines and growth factors, leading to fibroblasts migration and proliferation and in this way macrophages play an important role in tissue regeneration [90]. These macrophages are produced in response to IL-4 generated during innate or adaptive immune responses [85,91]. Regulatory macrophages also are produced within innate and adaptive immune

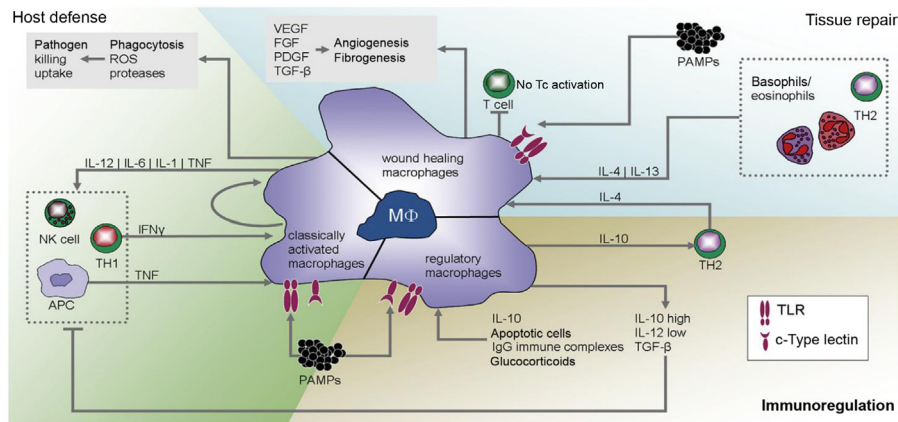


FIGURE 20.2 Macrophages base on different functions are classified into classically activated, regulatory, and wound-healing macrophages. Source: Reprinted from Franz S, Rammelt S, Scharnweber D, Simon JC. Immune responses to implants—a review of the implications for the design of immunomodulatory biomaterials. *Biomaterials* 2011;32 (28):6692–709 with the permission from Elsevier.

reactions, responding to a range of signals such as apoptotic cells, IL-10, glucocorticoids, and immune complexes [85,92]. Regulatory macrophages secrete high amounts of IL-10, a strong immunosuppressive cytokine, in order to restrict inflammation and down-regulate immune responses which is the principal duty of them [85,92,93]. The function of all three macrophage populations are engaged in an immune response, classically activated macrophages in the first phase and regulatory and wound-healing macrophages in resolution phase [94].

20.4.3 Adaptive immune response

Immune cell responses to a metal implant are either innate or adaptive immune responses. Adaptive immune response is required only when an antigen is presented to T cells. Activation of DCs has an important role in adaptive immune responses due to the initiation of antigen presentation and T cell activation, a complicated multiple procedure [95]. DCs present major histocompatibility complex (MHC) and costimulatory molecules at the implant site, leading to activate and expansion of the naïve T helper cells [96,97]. Induction of antigen-specific T cell tolerance, T cell energy, and T cell regulatory activation also are other immunoregulatory functions of DCs [98,99]. Tolerogenic DCs as a strong toll can limit the immune response and result in implant integration and wound healing at the biomaterial site [100]. A variety of cytokines and growth factors such as IL-6, IL-16, and $\text{TNF}\alpha$, hepatocyte growth factor and granulocyte colony stimulating factor in addition to IL-10 and $\text{TGF-}\beta$, have been found to produce tolerogenic DCs [99,101,102]. Biomaterials are supposed to activate DCs using PRRs and pathogen recognition signals [64] (Fig. 20.1B and E). The type of engaged PRR can determine promotion or inhibition of DC

maturation. Inhibition of DC maturation leads to tolerance, downregulation of the immune cells and limitation of inflammatory response [45]. Therefore, designing the surface chemistry of implants to induction of DCs with tolerogenic phenotype and modulating immune responses could make the biocompatibility better.

20.4.3.1 Sensitization

Metal ions can start both local and systematic hypersensitivity reactions. The immune reaction to metal ions is mainly believed to be a type IV or delayed hypersensitivity response involving the infiltration of T lymphocytes. In reactions related to type IV hypersensitivity, the released metal ions can act as allergens and become available as haptens, activating adaptive immune responses as the initial mechanisms. Since haptens are too small to elicit type IV hypersensitivity reactions, they bind to serum proteins and form hapten-protein complexes that are presented to T lymphocytes as antigens [16,103]. T lymphocytes, the key mediators of delayed hypersensitivity reactions, then secrete cytokine mediators to activate tissue macrophages leading to inflammation response. Additionally, metal ions can cause MHC molecule to be presented as foreign by making conformational changes [5].

It has been reported that the release of metal ions from alloys utilized in orthopedic devices, including titanium alloy, stainless steel, and cobalt–chromium result in type IV hypersensitivity reaction. There are some reports investigating metal allergy caused by implants used in fracture fixation or MoM hip replacements [104,105]. Failure of MoM joint replacements produce high amounts of nanosized wear particles. Corrosion of these products and further release of metal ions induce adverse tissue responses. High levels of cobalt and chromium in surrounding tissue, blood, and urine have been revealed in patients with MoM joint replacement [105,106]. Development of adverse tissue reactions surrounding MoM implants is specified with the formation of large cystic or noticeable bone loss. Large areas of necrosis and macrophage in addition to T and B lymphocyte and plasma cell infiltrates are normally observed histologically. The adverse response to MoM implants is characterized by infiltration of T lymphocytes, as histopathological evidence of a metal hypersensitivity [107,108].

20.4.3.2 Effects of metals in adaptive immunity

The effects of metal ions such as titanium, cobalt, and chromium ions on adaptive immune cells are reported by many studies. Several studies reported that micron-sized particles of titanium were ineffective on proliferation and IL-2 production from activated lymphocytes [109]. Nanoparticles of cobalt–chromium also did not activate DCs in vitro and decreased the proliferation of activated T lymphocytes [110]. According to another study, cobalt, chromium, and titanium ions with no cytotoxicity detection, prevent activation of T and B lymphocyte and the generation of inflammatory cytokines [111]. Apoptosis induction in lymphocytes was reported at higher concentrations of cobalt and chromium ions [112]. Cytotoxic effects of metal ions on human T lymphocytes with induction of apoptosis and cell necrosis but with no DNA damage also were found by other reports [113]. Inhibiting the effects of cobalt–chromium from the release of cytokines from T

lymphocytes and production of IgG from B lymphocytes, are more obvious than titanium particles [114]. Overall, most of the studies expressed that cobalt, chromium and titanium ions show suppressive effects on activation of lymphocytes and also revealed cytotoxic effects at higher concentrations. Reports on the function of metal ions with nanosized particles are relatively fewer than micron-sized particles. Brown et al. [115] showed that micron-sized particles of cobalt–chromium are phagocytosed by antigen-presenting cells and introduced to T lymphocytes by MHC II molecules, while nanosized particles lead to DNA damage through relatively unknown mechanisms.

20.5 Modulation of host response to implants

Implantation of most biomaterials initiates a host reaction with a cascade of events responding to adverse stimuli. Due to the release of wear particles and metal debris from implants, inflammatory reaction is initiated by tissue macrophages leading to repair tissue surrounding the implant. To achieve the survival of the organism, a balance between repair and inflammation is required. In an ideal situation, acute inflammation, prevention of chronic inflammation and fibrosis, repair of injured tissue, and biomaterial integration should be carried out [5,116]. Biologically inert biomaterial has been used for a long time to avoid immune response and cell-biomaterial interactions [117]. However, specific cell responses have been found to be favorable for implant integration and to develop implant function [118]. For example, cementless joint replacements always are not able to allow cells to adhere on implant and osseointegrate with the surrounding bone, thus may lead to implant loosening [119]. Development of the knowledge about immune responses to implants and healing process has developed immunomodulation of biomaterials, focusing on surface coating in the field of bone tissue engineering and tissue repair [45,116] (Fig. 20.3). It was reported by many studies that in modulation of innate and adaptive immune responses, the activity of NK cells was suppressed but macrophage activities continued unchanged [121].

Current available techniques in biomaterial designing involve passive modification of material surface through physicochemical properties or active modification with molecules designed to target cell behavior, producing specific patterns or coatings on the implant surface [45]. Upon implantation, the biomaterial surface is covered with a layer of host proteins that act as the binding site for macrophages and then make them able to signal in the immune cells. So, the protein layer can influence the attachment and survival of cells such as macrophages and subsequent inflammatory response to the implant [122,123]. The aim of passive modulation of the implant surface is to minimize macrophage adhesion and its fusion to FBGCs through surface modification [124,125]. Modulation of the implant through functionalization contains specific surface coatings and the bioactive molecules including adhesion sites, growth factors, antiinflammatory mediators, or drugs either alone or mixed [126] (Fig. 20.4).

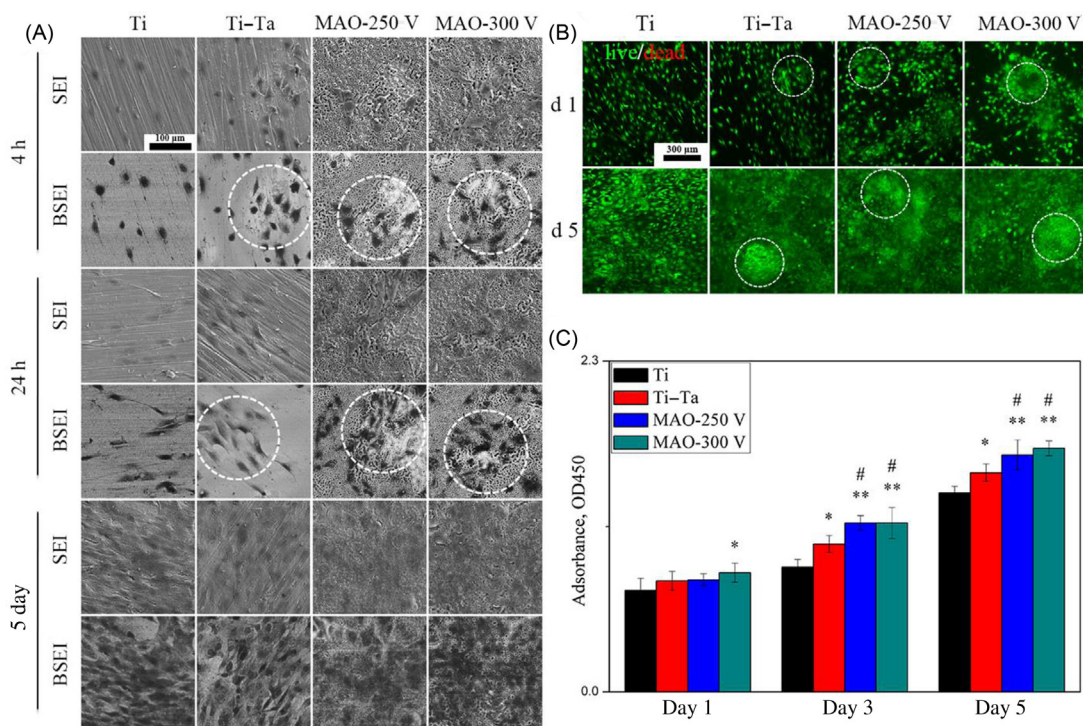


FIGURE 20.3 (A) SEM images showing the distribution, (B) fluorescent images of live/dead staining revealing viability, and (C) CCK-8 assay demonstrating the proliferation of human osteoblastic (SaOS-2) cells cultured on Ti, Ti–20%Ta metal–metal composite (referred as Ti–Ta), Ti–Ta surface modified with MAO at voltage of 250 (referred as MAO-250 V) and 350 V (referred as MAO-350 V) for different durations; * $P < .05$ and ** $P < .01$ compared with Ti, # $P < .05$ and ## $P < .01$ compared with Ti–Ta, and $P < .05$ and $P < .01$ compared to MAO-250 V. MAO treatment was employed to modify the surface of Ti–Ta composite as an implant material in order to improve cell adhesion, proliferation, and limit immune responses. MAO, Micro-arc oxidation. Source: Reprinted from Huang Q, Elkhoory TA, Xu S, Liu X, Feng Q, Wu H, et al. The osteogenic, inflammatory and osteo-immunomodulatory performances of biomedical Ti-Ta metal–metal composite with Ca- and Si-containing bioceramic coatings. *Colloids Surf B: Biointerfaces* 2018;169:49–59 [120] with the permission from Elsevier.

20.6 Conclusion

The aim of this chapter was to discuss currently used metallic implants in the body and to comprehend complex cellular responses of the host to implants. Biomaterials induce the host immune responses and are a major consideration in design of immunomodulation. Biological responses to implants at the biomaterial site can also induce immune responses leading to degradation or failure of the implant. Systematic and/or local modulation of materials to stimulate specific cell responses can develop new potentials for treatments, applications, and implant integration that may improve the function and longevity of implants. Designing of implant surfaces through altering physicochemical properties or actively modification of surfaces are commonly used methods to control the adverse cellular responses to biomaterials. It

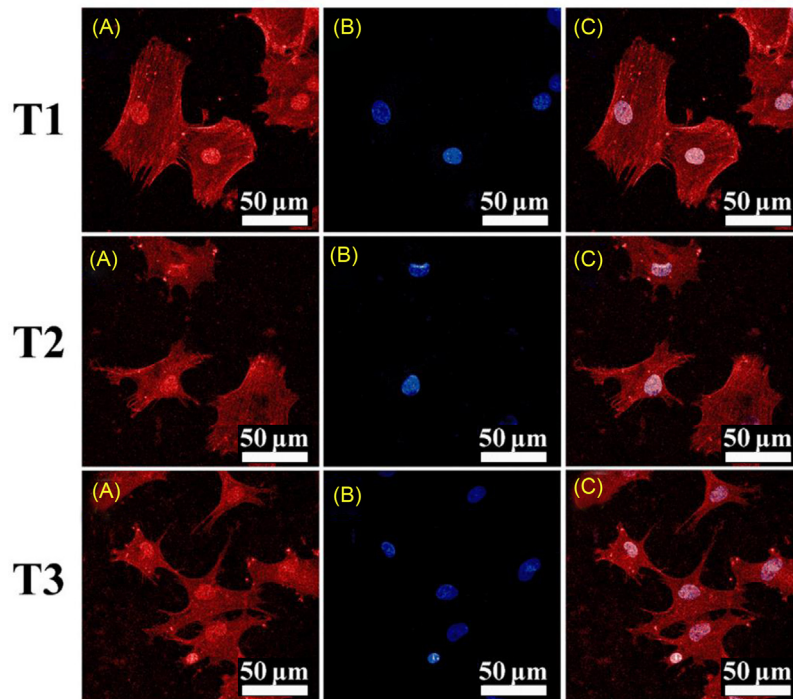


FIGURE 20.4 CLSM images of rBMSCs attachment after culturing on different HA coatings on Ti–6Al–4V implant surface for 6 h. (A) actin stained with red, (B) cell nuclei stained with blue, (C) merged A with B. Nanostructured and high crystallinity HA coatings on Ti–6Al–4V implant were used to improve cell adhesion, proliferation, and osseointegration. The plasma-sprayed HA coating with the addition of hydrothermal treatment in Milli-Q water at 180°C and 24 h and 0.2 mol/L Na_3PO_4 solution at 180°C and 24 h, were labeled T2 and T3, respectively. The plasma-sprayed HA coating without hydrothermal treatment was labeled T1. T2 and T3 demonstrated better cell adhesion and cell spreading with more typical filopodia attachment. CLSM, Confocal laser scanning microscopy; HA, hydroxyapatite; rBMSCs, rat bone mesenchymal stem cells. Source: Reprinted from Xia L, Xie Y, Fang B, Wang X, Lin K, et al. *In situ modulation of crystallinity and nano-structures to enhance the stability and osseointegration of hydroxyapatite coatings on Ti-6Al-4V implants*. *Chem Eng J* 2018;347:711–20 [127] with the permission from Elsevier.

should be noted that there is still complexity in material science that could be developed to coordinate the biological complications at the molecular level.

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Cellular response to nanobiomaterials

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21.1 Introduction

Nanotechnology is an evolving field which plays key roles in the development of several important products and processes based on tiny materials called nanomaterials. Nanomaterials or nanostructures can be defined as any material that has one or more external dimension in the nanoscale of 1–100 nm [1]. Nanomaterials used in biological or biomedical applications are generally referred to as nanobiomaterials. Nanobiomaterials show higher physicochemical properties per unit weight compared to their bulk forms. These characteristics make them suitable for several applications including several fast-moving consumer goods products and medical devices. Inorganic nanomaterials such as various metal/metal oxide nanoparticles and organic nanomaterials such as carbon nanotubes (CNTs), graphene, C60 fullerenes nanocrystals, and carbon quantum dots (QDs) are used as nanobiomaterials. Earlier, the general assumption regarding the effect of nanomaterials on cells and cellular components was vague. Several nanostructured materials were considered as biologically inert and safe for daily and even medical applications. However, results from various applied biomaterial, nanomedicine, and nanotoxicology research indicate that in addition to the beneficial effects, nanomaterials can generate a diverse range of adverse effects [2]. Most of these adverse effects at cellular level are due to the generation of reactive oxygen species (ROS) which creates oxidative stress. ROS can induce the expression of several signaling molecules that are important in inflammatory pathways [3]. Non-ROS-based effects of nanobiomaterials on biological system have also been reported [4,5]. Such effects are mainly due to several factors such as shape, size, surface chemistry, surface charge, and the metal ion release from the nanoparticles [6,7]. In most of the cases, the window between the therapeutic and cytotoxic level of nanobiomaterials is very narrow and varies from cell to cell. However, regardless of the huge amount of data scattered in scientific literature, the understanding of nanobiomaterials–cellular interactions remains to be reorganized.

Physical and chemical properties of nanobiomaterials including size, shape, and surface properties have important roles in deciding the interaction with cells and organelles. Surface modification of the nanobiomaterials can play a significant role in the cellular response since it can influence the nature of surface adsorbed proteins. Surface topography, surface chemistry, and surface mechanical properties are the major surface factors that play key roles in nanobiomaterial–cell response [8]. These properties of nanobiomaterials influence the physiological processes of cells including phagocytosis, renal excretion, and several biological signaling pathways [9]. The interaction of certain inorganic nanoparticles with cells and its individual components can produce pronounced cytotoxic effects. However, on the positive side, the cytotoxicity of certain nanoparticles is exploited to kill pathogens and malignant cells which have a great potential in healthcare. For a clear understanding of the harmless and harmful dose of nanobiomaterials and drawing a sharp line between therapeutic window and cytotoxic concentration, substantial advancement should be achieved in order to understand the relevant interactions at nanobiomaterial–cell interfaces [10]. Thus, various nanobiomaterial–cellular interactions and the resulting cellular responses highlight their evolving opportunities and challenges. The consequence of interaction of nanobiomaterials on cells, their components and functions, at cellular and molecular level, could help recognize the essential requirements on the use of nanobiomaterials for day-to-day and medical applications.

21.2 Factors affecting nanobiomaterial–cell interactions

There are several factors at the nanomaterial's point of view that dictate the interaction between nanobiomaterials and cellular components. The outcome of such interactions can be either beneficial or nonbeneficial. Some of such factors are explained in the upcoming sections.

21.2.1 Chemistry of nanobiomaterials

Chemistry of the bulk of nanobiomaterial or the specific surface chemistry of nanobiomaterial can influence cellular responses such as proliferation, adhesion, spreading, differentiation, apoptosis, and necrosis of various types of cells [11]. Nanoparticles produced from natural polymers are generally cell friendly. For example, nanoparticles produced from chitosan have antimicrobial capability, biocompatibility, and high reactive adsorptive ability [12]. These favorable characteristics are ascribed to the presence of functional groups such as hydroxyl, amino, and acetamido groups. Nanoparticles produced from other natural polymers such as alginate, gelatin, carboxymethyl cellulose, etc. are also cell friendly [13]. Synthetic polymers such as polycaprolactone, polylactide-*co*-glycolide, polylactic acid, and their degradation products, etc. are nontoxic to the cells which make the nanoparticles based on them safe candidates for drug delivery systems [14]. Since elemental gold [15] and platinum are relatively nontoxic to the normal mammalian cells, nanoparticles based on them are safe despite the effects associated with the nanoscience of them [16]. The toxicity associated with the specific element itself can be the primary reason for the cytotoxicity associated

with the nanoparticles. For instance, nanoparticles based on cadmium and lead are generally toxic to the cells unless they are fully coated by a stable coating of nontoxic material [17]. Cellular responses against nanomaterials have been related to both specific (e.g., specific chemical molecules) and nonspecific (e.g., presence of functional groups) surface chemical properties of nanobiomaterials. Specific chemical properties of nanobiomaterials play a significant role in the systemic toxicity. However, nonspecific chemical properties such as charge and wettability have been shown to influence local responses such as cell adhesion or phenotype [18,19].

Dissolution of metallic nanoparticles has a significant role in the toxicity at cellular and systemic level [20]. The dissolution of nanobiomaterials mainly relies upon the metal solubility within a given microenvironment and the size, shape, and surface topography. Solubility of nanobiomaterials directly impacts the cytotoxicity of several metal oxide nanoparticles. Nanomaterials based on more soluble compounds like zinc oxide (ZnO) show a more pronounced cytotoxicity on mammalian cells compared to less soluble ones like TiO₂ [21]. Metal ions generated from such nanomaterials might be the reason for the higher adverse effects related to more soluble ones. Moreover, the release of Ag⁺ and Cu²⁺ ions from silver and copper nanoparticles and the binding of the surface atoms to electron donor groups of cell wall components containing oxygen, sulfur, or nitrogen is the major reason for their antimicrobial properties [22]. Therefore, since chemical nature of the material used for the synthesis and/or functionalization can have a direct influence on the interaction of the obtained nanoparticles and the mammalian cells in contact, the chemistry of biomaterials must be carefully examined before designing or synthesizing nanobiomaterials.

21.2.2 Size of nanobiomaterials

One of the most important factors that plays a critical role in cell–nanobiomaterial interaction is the size and size distribution of the nanobiomaterial itself. The size plays a key role in the kinetics of drug release, biodistribution, cellular uptake, and toxicity of nanobiomaterials [23]. Nanoparticles are mostly not recognized as a foreign bodies by macrophages [24] but microparticles are recognized as taken up by reticuloendothelial systems [25]. Macrophage uptake of nanobiomaterials is also dependent on particle size and relatively large nanoparticles are only recognized by them [26]. In addition to the cellular uptake efficiency, the size of the nanobiomaterials also affects their internalization kinetics and distribution [27,28]. Nanobiomaterials enter into the cells through cell membrane pores [24]. The large surface area of the small nanoparticles facilitates the easy diffusion of them into the cells [29]. For example, 30 nm sized single-walled CNTs (SWCNTs) are internalized much higher than 50 nm ones [30]. Among gold nanoparticles with 14, 50, and 74 nm size, ones with 50 nm were the most efficiently internalized by HeLa cells [31]. This indicates that there might be an optimal size for efficient nanomaterial uptake into the cells [23]. Irrespective of functional groups on the surface, smaller nanoparticles show a higher cellular uptake in cancer cells [32]. For example, the length of CNTs determines the cellular uptake behavior. Multiwalled CNTs (MWCNTs) with submicron length have shown to possess more cell penetration capacity compared to longer ones [33]. In addition, the size of nanomaterials influences the binding and activation of several important cell membrane receptors and regulates protein expression [34].

Understanding the effect of the shape and size of nanobiomaterials on their interactions with cellular components is highly important due to its influence on cytotoxicity [35]. Polydispersity index of nanomaterials is another important factor to be considered since different sized particles of the same material might display different cellular response. Moreover, during the *in vitro* and *in vivo* studies, nanostructures may agglomerate to form larger particles with different sizes that may influence the interactions and cellular response [36]. Depending upon the nanomaterial size, the mechanism of internalization may vary. For example, clathrin-coated pits regulate the penetration of particles with <200 nm diameter while 500 nm ones internalize through caveolae-mediated process [37]. Interestingly, nanostructures with 50 nm diameter can target mesenchymal stem cells with clathrin and internalize independently from endocytosis [38].

21.2.3 Shape of nanobiomaterials

Particle shape is another important physical parameter with significant influence on nanobiomaterial–cell interaction. Particle shape influences uptake, distribution, and cellular functions [39]. Unlike spherical nanoparticles, elongated ones generally show a higher uptake due to their potential to effectively bind on the cells [40]. This is because, the curved surfaces of spherical particles provide a smaller number of binding sites to interact with the cell surface. In contrast, the elongated nanostructures have a higher surface area to volume ratio that ensures the effective interaction with cell surface [41,42]. For example, rod-shaped nanostructures were internalized by HeLa and Caco-2 cells more effectively than lower aspect ratio nanoparticles [43]. Moreover, the rod-shaped nanomaterials have shown higher uptake by endothelial cells [44]. However, compared to rod-shaped nanostructures, HeLa cells internalized discoid particles more effectively [45]. Higher cellular internalization was detected for cylinder-shaped nanomaterials than spherical ones [46]. Generally, nanostructures with sharp shapes can pierce the cell membranes and effectively internalize. The exocytosis of high aspect ratio nanoparticles was lower than the spherical ones [47]. Cytotoxicity of graphene-oxide (GO) nanoribbons was significantly higher than GO nanoplatelets [48]. However, spherical polymer nanoparticles and their mechanically deformed quasiellipsoidal nanostructures with different aspects ratios showed contrasting internalization trend [49]. Spherical nanoparticles exhibited higher uptake than the nonspherical counterparts. Gold nanoparticles with triangular shape showed higher cellular internalization than the spherical ones in HeLa cells [50]. Among methylpolyethylene glycol decorated gold nanoparticles with star, rod, and triangle shapes; star, rod, and triangle-shaped ones showed the lowest, medium, and highest cellular uptake, respectively [51].

21.2.4 Surface topography and stiffness of nanobiomaterials

Both topography and stiffness of nanomaterials can influence their interaction with cellular components and resulting cellular responses. Topographical patterns and their dimensions are critical for determining the interaction of nanomaterials with cellular structures [36]. For instance, unlike large surface patterns [52], subnanoscale or nanoscale surface patterns can influence the cellular response [53]. In addition, nanotopographical

features can influence cell adhesion [54] and differentiation [55]. Despite the size of the pattern, pattern of organization of them on the surface of nanomaterial can also produce variation in cellular response [56]. Matrix stiffness and elasticity of the nanomaterial can also influence the interaction and resulting response [57]. Huang et al. used polyacrylamide nanoparticles with tunable stiffness as model substrates to demonstrate the effect of nanomaterial stiffness and cellular uptake of them by bovine aortic endothelial cells [58]. They observed that a stiffer nanomaterial can undergo higher cellular uptake on a per cell basis, but a lower internalization per unit cell membrane area.

21.2.5 Surface charge

Surface charge of nanomaterials influences their adhesion to the cell membrane, cellular internalization, and resulting cytotoxicity. Thus, surface charge of nanobiomaterials is an important factor that should be considered to facilitate the successful cellular interaction and cellular internalization. The overall surface charge of the nanoparticles is generally expressed as zeta potential [59]. Since the plasma membrane shows overall negative charge, cationic nanomaterials are supposed to be electrostatically attracted towards it and internalized by the cells. Despite these theoretical considerations, nanomaterials with negative surface charges also enter in cells by passing the anionic cell membrane [60]. However, neutrally charged nanomaterials show low interaction with cells and internalization [23].

21.2.6 Functional groups of nanobiomaterials

Surface functional groups of nanomaterials can influence the cell–nanobiomaterial interactions. Functional properties of nanostructures can be changed by covalently functionalizing with different molecules. The surface of nanobiomaterials can be decorated with biomacromolecules to exploit the specific interaction of cell surface ligands with the nanobiomaterials. Nanobiomaterials with suitable surface modification could facilitate safe membrane penetration and cellular internalization. For instance, gold nanoparticles conjugated with oligonucleotides exhibited desirable stability in biological environments, cellular penetration, and enhanced target binding [61]. In order to achieve specific interaction and binding of nanoparticles to cell surface receptors, targeting ligands such as peptides, proteins, small molecules, antibodies, and nucleic acids can be attached on the surface of nanomaterials [62]. Chain length of functional molecules used to link the targeting antibody considerably affects the targeting capacity of nanomaterial. For instance, it was evident when gold nanoparticles were decorated with high molecular weight polyethylene glycol (PEG) and conjugated a monoclonal targeting antibody [63]. Surface functionalization with low molecular weight PEG could reduce the development of protein corona upon contact with serum [63]. Nanoparticles can be coated with PEG to avoid nonspecific cellular interactions due to its ability to resist protein adoption. Coating with PEG can prevent the self-aggregation of nanoparticles and reduce the nonspecific uptake by macrophages [64]. It is important to note that those nanoparticles with exposed PEG functional moieties on the surface show less protein binding and cell interaction, irrespective of

overall functionalization [36]. Compared to PEG-coated analogs, hydroxyl surface functionalized QDs have a significant reduction in nonspecific cellular binding [65].

21.2.7 Hydrophobicity/hydrophilicity of nanobiomaterials

Surface hydrophobicity/hydrophilicity also can influence the interaction between cells and nanobiomaterials. Nanobiomaterials with hydrophilic surfaces exhibited longer circulation time due to reduced phagocytosis [66]. Unlike hydrophobic surfaces, macrophages grown over hydrophilic titanium surfaces showed the presence of antiinflammatory activation comparable to the antiinflammatory M2-like state [67]. Similarly, surfaces bearing moderately hydrophilic molecules could promote cell adhesion and proliferation [68]. Nanoparticles functionalized with $-\text{OH}$, $-\text{NH}_2$, or $-\text{COOH}$ groups were tested for their cytotoxic effects on cell

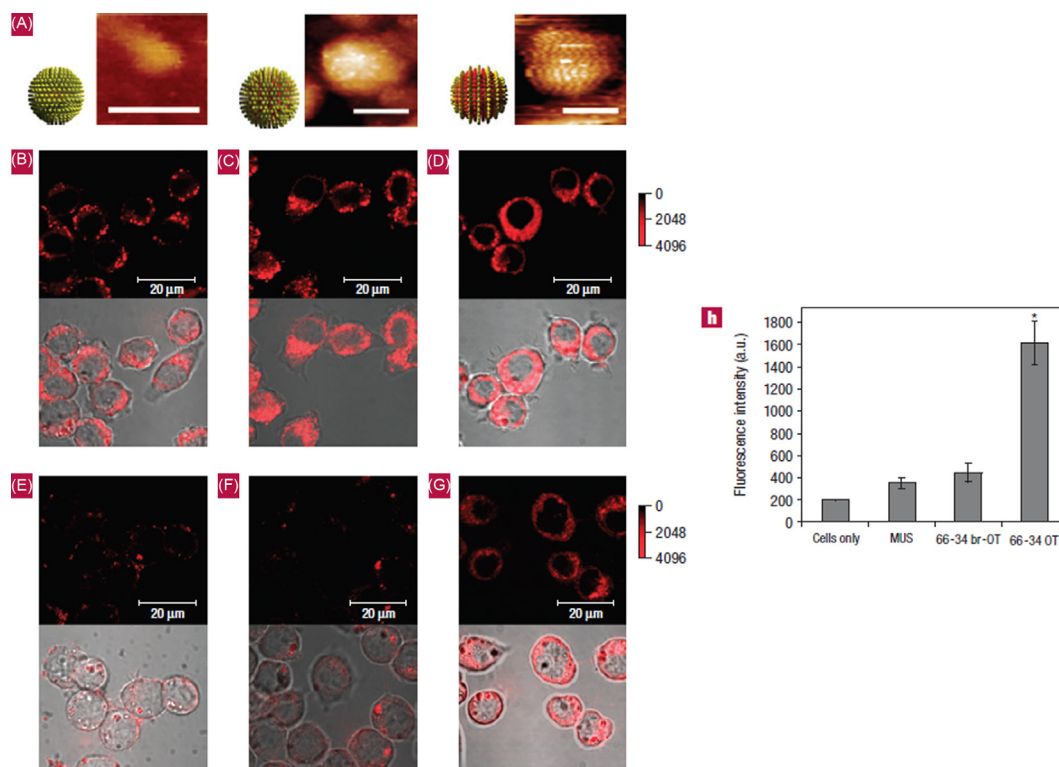


FIGURE 21.1 Subcellular localization of nanobiomaterials with ordered arrangements of hydrophobic and hydrophilic functional groups in cells. Schematic diagrams of the ligand shell structure of the nanoparticles and their STM images (scale bars 5 nm) (A). BODIPY fluorescence [upper panels with an intensity scale bar (a.u.)] and bright-field/fluorescence overlay (lower panels) images of dendritic cells incubated with MUS (B and E), 66-34 br-OT (C and F) or 66-34 OT (D and G) nanoparticles for 3 h in serum-free medium at 37°C (B–D) or 4°C (E–G). Source: Reproduced with permission from Verma A, Uzun O, Hu Y, Hu Y, Han HS, Watson N, et al. Surface-structure-regulated cell-membrane penetration by monolayer-protected nanoparticles. *Nat Mater* 2008;7:588–95. <https://doi.org/10.1038/nmat2202>.

lines such as Lewis lung carcinoma, B16F10 melanoma, JHU prostate cancer cells, and 3T3 fibroblasts. Interestingly, $-\text{NH}_2$ and $-\text{OH}$ group functionalized nanoparticles showed higher cytotoxicity than $-\text{COOH}$ functionalized ones with significant cell membrane damage [69].

Fig. 21.1 shows the difference in cellular behavior between nanoparticle with varying hydrophilicity [70]. Hydrophilic sulfonate ligand-bearing nanoparticles (MUS) that were internalized by dendritic cells showed punctate fluorescence patterns showing the successful endosomal internalization (Fig. 21.1B). A similar pattern was observed on disordered mixed monolayer with sulfonate and methyl-head groups (66-34 br-OT). They also exhibited a low level of background fluorescence in some cells (Fig. 21.1C). However, nanoparticles with a “striped” nanoscale organization have a similar ratio of methyl and surface sulfonate groups (66-34 OT) as in 66-34 br-OT and were detected in cells as a diffuse pattern of intracellular fluorescence due to the internalization by endocytosis (Fig. 21.1D). Thus, the nanoparticles generally enter in cells through two mechanisms based on hydrophilicity: energy-dependent (endocytosis) and energy-independent processes [70].

21.3 Various interactions between nanobiomaterials and cells

Several approaches such as modifying the surface topography [71], chemical functionalization [72], and controlling the material stiffness [73] were tried to rationally design nanobiomaterials to attribute them with capabilities to specifically influence cellular response [74]. The final goal in designing such structures is to control nanobiomaterial–cell interactions and attain a desired cellular response. Such interactions of nanobiomaterials with cells and cellular components are crucial in several biomedical applications such as bioimaging, drug delivery, phototherapy, wound healing, and tissue engineering.

Although the goal of designing nanobiomaterials is to get a desired outcome, their interaction with the cells can be either beneficial or deleterious to the biological system. When nanobiomaterials enter the tissues, they will interact with the ECM proteins and form a corona around the nanoparticles. Such corona can prevent agglomeration between nanoparticles, reduce toxicity, and prevent them from entering the cells. However, corona formation may affect the physicochemical properties of the nanobiomaterial. Another example is the generation of ROS by metal oxide nanoparticles. Under optimum concentration, ROS can support growth factor secretion, tissue regeneration, and wound healing. However, a higher quantity of such nanoparticles can generate excess ROS which produces deleterious effects such as oxidative damage of proteins, cell death, and DNA damage. The following sections provide a discussion regarding various types of interactions that occur between nanobiomaterials and various cellular components.

21.3.1 Nanobiomaterial–ECM interactions

The ECMs are multicomponent frameworks that surround the cells in tissues and participate in the implementation of several cell biological functions such as cell–cell communication and nutrient transport [75]. Moreover, they can act as a scaffold that provides strength and shape to the tissues in vivo. The major components of ECM are fibrillar proteins such as laminins and

collagens, nonfibrillar proteins such as glycoproteins and proteoglycans (PGs). Structural rigidity of ECM is mainly due to the collagen fibers, whereas the flexibility is provided by elastin. It also contains several glycosaminoglycans (GAGs) [76]. Hydrogel-like texture of ECM is provided by PGs and protein-linked GAGs. ECM possesses a net negative charge due to the presence of GAGs which are rich in sulfate- and carboxyl functional groups. Cell adhesion and proliferation is manifested by several types of integrins and other matrix proteins. Moreover, any change in the ECM organization can greatly impact cellular signal transduction events [77].

In order to reach the cells, interact with them, and produce a biological response, nanobiomaterials must be able to travel through ECM. Although there are several barriers exist in the ECM for diffusing nanomaterials; major factors that affect the mobility are hydrodynamic diameter and surface charge of the nanomaterials [78]. Owing to the mesh-like structure, ECM tightly regulates the nanoparticle transport across it [79]. The straining property of ECM is mainly depending on the density of all the component biomacromolecules of ECM. ECM allows the passing of particles smaller than the mesh space whereas larger particles are rejected (Fig. 21.2). Collagen fibrils possess an interfibrillar spacing of 20–40 nm and ECM generally allows the passing of smaller particles [81]. Thus, nanoparticles having less than 20 nm diameter can pass ECM.

In addition to the size filtering, both the charge of nanomaterials and ECM components influence the trafficking of nanoparticles across ECM by a process termed as interaction filtering (Fig. 21.2) [82]. Generally, positively charged nanoparticles are engulfed by cells more than negatively charged ones [83]. However, citrate-gold nanoparticles were attracted by hyaluronan, negatively charged GAG of ECM [84]. High affinity binding of citrate-coated superparamagnetic iron oxide particles was hindered when GAG synthesis was inhibited [85]. This implies that the interaction between negatively charged GAGs and the citrate-coated nanoparticles played an important role in the cellular binding of nanoparticles.

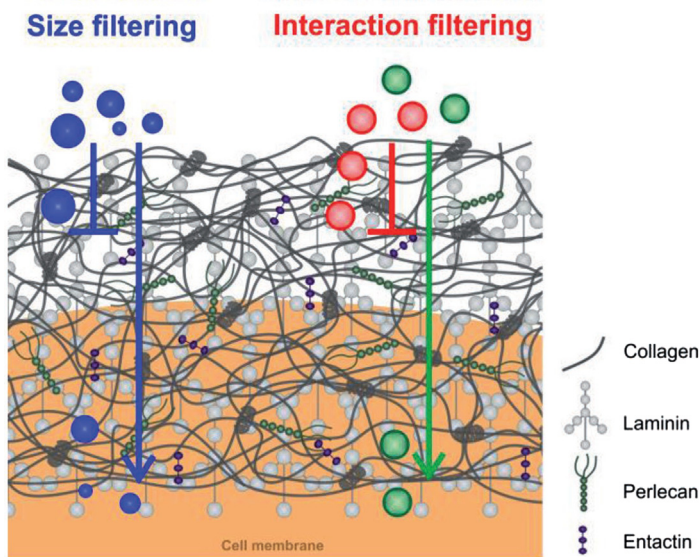


FIGURE 21.2 Scheme showing the size of nanomaterial and interaction with the ECM components and their trafficking across cell membrane [80].

Cell density also plays an important role in ECM-nanomaterial interaction. Nanoparticle uptake is generally affected when the cells are tightly packed. In cell junctions where the inter-membrane distance is as short as a few nanometers, interactions between adjacent cells trap nanoparticles in ECM and keep them out of target cells [86]. This is a big challenge when nanoparticles are used in cancer treatment [87]. Nanomaterials targeted to the central nervous system are also prone to this issue. Blood–brain barrier (BBB), which is a tight junction between blood circulation and brain tissue regulates the entry of particles and molecules to brain. High electrical resistance and low permeability of the brain endothelium is due to the presence of tight junctions formed by the tight arrangement of the microvascular brain endothelial cells. Transmembrane proteins like occludin and claudins along with actin cytoskeleton form the major macromolecular components of BBB [88]. Interaction of ECM components of BBB with nanobiomaterials plays a significant role on the outcome of the respective brain-targeted nanomaterial therapy. Surface modification of nanomaterials are being tried to overcome the barrier effect of BBB. For example, paclitaxel-loaded PEG–poly(D,L-lactide-*co*-glycolide) (PLGA)-block copolymer nanoparticles showed 100-fold faster brain diffusion than similar particles without PEG coatings [89].

Certain nanoparticles can alter the ECM structure also. For instance, nanoparticles can induce the degradation of ECM by activating inflammatory signaling pathway [90]. Nanoparticle-induced upregulation of matrix metalloproteinases expression results in the disorganization of ECM proteins [90]. Carbonaceous nanobiomaterials enter the cell by adhesive interactions [91]. Pure CNTs can generate ROS in cells due to their high surface area and the specific surface reactivity [92,93]. ROS generation can damage component proteins of ECM.

Tissue engineering is a rapidly developing field which involves the development of living tissue substitutes that can repair, regain, or replace the functions of damaged tissues [94,95]. Tissue engineering scaffolds are ECM mimicking porous biodegradable support materials used to grow cells for tissue engineering purposes [96,97]. An ideal scaffold should replicate the native *in vivo* environment as much as possible [98]. Thus, artificial nanofibrous membranes showing the topographies of the native ECM have great potential as scaffolds in tissue engineering [99]. Electrospinning is a widely used technique to fabricate nanofibrous scaffolds [99]. The diameter of such electrospun fibers usually lies in sub-micron range, which is comparable to the nanoscale architecture of native ECM [100]. Mo et al. developed ECM mimicking poly(L-lactide-*co*- ϵ -caprolactone) [P(LLA-CL)] with L-lactide to ϵ -caprolactone based nanofibers and characterized its effect on smooth muscle cells and endothelial cells [101]. Both types of cells could adhere and proliferate well on the P(LLA-CL) nanofiber scaffolds. Nanofibrous scaffolds based on natural polymers such as collagen and chitosan can closely mimic native ECM both structurally and functionally [102]. Various nanoparticles such as ZnO [103], TiO₂ [104,105], cerium oxide [106], yttrium oxide [107], and silver nanoparticles [108] can be incorporated in tissue engineering scaffolds to improve the physicomaterial and biological performance.

21.3.2 Nanobiomaterial–cell membrane interaction

The cell membrane acts as the first interface between the cells and the nanobiomaterials. The phospholipid bilayer assembly of the cell membrane maintains the stability and integrity of the

internal environment of the cells. It is highly imperative to understand the biophysical interactions of nanobiomaterials with cell membranes to minimize the nanotoxicity and improve the effectiveness of nanodrug formulations. Upon contact, nanobiomaterials can induce the deformation of lipid membranes leading to their uptake [109]. Upon contact with cell membranes, certain nanobiomaterials can disturb the phospholipids bilayer and disorganize component membrane proteins [110]. In addition, nanomaterials can induce the formation of “holes” in the cell membranes. Holes in cell membranes could result in the leakage of intracellular macromolecules and produce cytotoxicity. Nanomaterials with surface positive charges are more likely to generate holes than negatively charged and neutral ones. Several factors such as size/shape of the nanomaterials, nanomaterial-membrane adhesion energy, net charge, and membrane’s mechanical property can influence the extent of nanobiomaterial–cell membrane interactions [111,112]. Cationic nanomaterials can produce an attractive interaction with negatively charged cell membranes and results in their rapid uptake with possible membrane damage [113]. On the other hand, anionic nanomaterials are less harmful to the cell membranes with similar net charge. Cationic lipid/DNA complexes (lipoplexes) are internalized by cells following the interaction of the cationic lipopolyamines with membrane PGs [114]. This can happen through endocytosis or direct penetration of the cell membrane without the influence of possible receptor-based interaction [109]. Fig. 21.3 explains the possible response by cell membranes upon contact with nanoparticles. If the adhesion energy between nanomaterial and the cell membrane is not strong enough, the nanomaterial will undergo Brownian collisions with the cell membrane without adhering to it. If adequate adhesive energy is available, nanomaterial can adhere on the membrane and can be partly covered by the lipid bilayer. At optimum conditions, the adhered nanoparticle can be entirely engulfed by the cell membrane. Later, these membrane-bound particles separates from the internal surface of the cell membrane into the cytoplasm by creating a transient pore in the cell membrane [115,116].

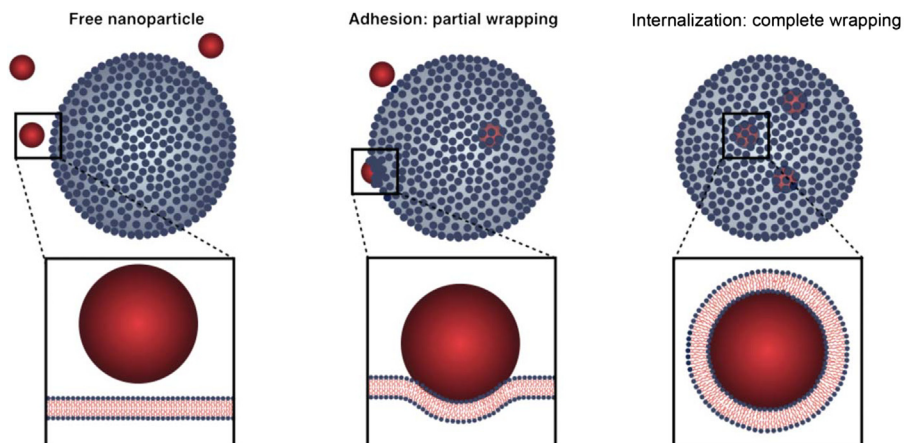


FIGURE 21.3 Three possible steps of nanomaterial–cell membrane interaction: free nanomaterial in the environment, nanomaterial adhesion to cell membrane, and complete covering by the cell membranes and uptake. Source: Reproduced with permission from Contini C, Schneemilch M, Gaisford S, Quirke N. Nanoparticle–membrane interactions. *J Exp Nanosci* 2018. <https://doi.org/10.1080/17458080.2017.1413253>.

A study using polystyrene nanoparticles of different surface chemistry and sizes indicated that 20-nm plain nanoparticles cause condensation of the cell membrane due to their diffusion into the liquid expanded phase of the cell membrane [117]. Plain 60-nm nanoparticles demonstrated the presence of a disrupted monolayer, due to the binding and the displacement of lipids at the interface resulting in the destabilization of the membrane. However, development of a “protein corona” on such plane nanomaterial surface may influence the nanomaterial–cell membrane interaction [118]. The nature of biomolecules in the corona plays a significant role in the specific recognition by the cell membrane receptors and results in regulated uptake [119]. Poly-L-lysine functionalized PLGA nanomaterials have shown five-fold higher force of adhesion with the membrane and which resulted in their rapid internalization than unmodified nanoparticles [120]. Cells incubated with functionalized nanoparticles showed wrinkles on the cell surface probably due to the internalization. Metallic nanoparticles also show size-dependent membrane–nanoparticle interactions. Gold nanoparticles with 20 nm could be internalized in cytosolic vacuoles whereas 70 nm ones were restricted by the cell membrane [121]. Copper nanoparticles are more cell membrane damaging than its oxide form, copper oxide (CuO) nanoparticles [122].

Assessing the interaction of nanomaterials with the target cell membrane and their internalization is highly important to improve the efficiency of nanomedicines. Various approaches including functionalization with specific molecules such as antibodies are tried to improve the interaction of nanomaterials with cell membranes. Adhesion between Triptorelin (a luteinizing hormone releasing hormone agonist)-conjugated PEG-coated magnetite nanoparticles (Triptorelin-MNPs) and breast cancer cells, showed that Triptorelin-MNPs have a 14-fold greater work of adhesion to breast cancer cells than to normal breast cells [123]. Such cell membrane targeted, and tumor-specific magnetic nanoparticles have a great potential as MRI contrast agents and drug delivery systems.

The interactions of mesoporous silica nanoparticles (MSNs) with various particle sizes and surface features with human red blood cells (RBCs) demonstrated that small MSNs (~ 100 nm, s-MSN) can adsorb on the cell membranes of RBCs without affecting the membrane organization [124]. However, adhesion of large MSNs (~ 600 nm, l-MSN) to RBCs resulted in the spiculation of RBCs and subsequent hemolysis. Normal biconcave shape was observed on RBCs incubated with s-MSN and control RBCs (Fig. 21.4A and B). However, local membrane deformation and particle encapsulation by several RBCs were observed on those treated with l-MSNs (Fig. 21.4C) with an echinocytic (spiculated) shape alteration and a considerable reduction in the surface area to volume ratio.

Carbon-based nanostructures such as fullerenes [125,126] and CNTs [127,128] can enter cells through the penetration of cell membrane [129,130]. Such materials enter into cells through endocytosis or spontaneous insertion and penetration across the membrane [131]. CNTs may cross the cell membrane by a lipid-mediated mechanism [132] which may involve landing, piercing, and subsequent uptake [133,134]. However, Pluronic-stabilized SWCNTs improved its interaction with cell membrane and increased the uptake [135]. Small graphene nanosheets are able to translocate across lipid bilayers [136]. Local piercing of membrane starts at the sharp edges of graphene and propagates along the remaining part. GO can produce profound plasma membrane (PM) ruffling and shedding in rat basophilic leukemia cells [137]. Carboxyl functionalization of graphene can reduce the strong hydrophobic interaction with cells membranes and the resulting cytotoxic effects

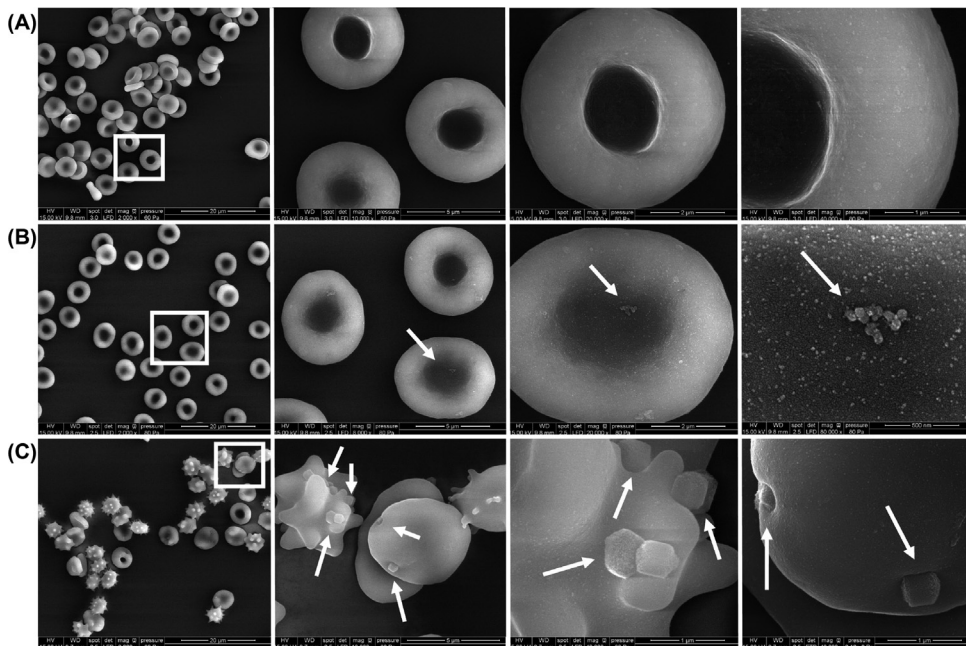


FIGURE 21.4 SEM images of RBCs treated for 2 h at room temperature with (A) control (PBS), (B) s-MSN (100 $\mu\text{g}/\text{mL}$), and (C) l-MSN (100 $\mu\text{g}/\text{mL}$). The nanoparticles attached on the cell surface are visible. Magnification of images increase from left to right. RBC, Red blood cells. PBS, phosphate buffered saline. Source: Reproduced with permission from Zhao Y, Sun X, Zhang G, Trewyn BG, Slowling II, Lin VSY. Interaction of mesoporous silica nanoparticles with human red blood cell membranes: size and surface effects. *ACS Nano* 2011;5:1366–75. <https://doi.org/10.1021/nm103077k>.

[138]. Internalization of graphene-like materials by cell membranes is usually initiated at the corners or irregular sides [136]. Such local piercing by these sharp protrusions avoids the high energy barrier and facilitates smooth propagation and internalization along the extended graphene edge.

21.3.3 Nanobiomaterial–cytoskeleton interactions

After penetrating the cell membrane, nanobiomaterials within the cytoplasm may adhere and interact with the cytoskeleton. Cytoskeleton is an interconnected system of filamentous proteins such as actin filaments, microtubules (MT), and intermediate filaments (IF) along with several regulatory proteins. Cytoskeleton helps to resist cellular deformation and provides the structural integrity and stability to the cells and has an important role in cell division and cellular movement [139]. It also transports intracellular cargo to various organelles of the cells. In eukaryotic cells, the cytoskeleton is composed of three filamentous proteins, namely MT, IF, and actin microfilaments (F-actins) [140]. IF are composed of different proteins such as vimentin, keratins, neurofilament proteins, nestin, and nuclear lamins [141]. Possible physical and chemical interactions between nanoparticles

and the cytoskeletal proteins determine the effect of nanoparticles on cytoskeletal integrity. Generally, nanomaterials interact with these structural proteins through van der Waals, electrostatic, hydrogen bonding, and hydrophobic interactions. MT can actively transport nanomaterials carrying vesicles such as internalized QDs present in lysosomes or endosomes [142]. The presence of intracellular tubulin in the protein corona formed around SiO₂ nanoparticles indicates that nanomaterials interact with cytoskeletal proteins [143]. Upon treatment with Si/SiO₂ QDs, the cell morphology was affected due to the disruption of the actin filaments [144]. Duan et al. showed that SiO₂ nanoparticles resulted in the disruption and disorganization of F-actin fibers in primary human umbilical vein endothelial cells (HUVECs) [145]. However, SiO₂ nanoparticles were rapidly internalized by differentiated THP-1 macrophages without affecting the microtubular network [146].

Interaction between citrate-coated silver nanoparticles and cytoskeletal proteins resulted in an increase in zeta potential, increase in hydrodynamics size, and a red-shift of the plasmonic band of silver nanoparticles [147]. Such interactions also result in the changes of the secondary structures of cytoskeletal proteins such as actin and tubulin. Silver nanoparticles also can induce changes in cytoskeletal integrity, including the increase of F-actin fiber formation and the variation of cell polarity [148]. Moreover, loss of cytoskeleton components such as F-actins and β -tubulins was observed on silver nanoparticle-treated rat cortical neurons [149]. Gold nanoparticles also can affect cytoskeletal integrity due to the inhibition of MT polymerization and the aggregation of tubulin heterodimers [150]. Other studies demonstrated that gold nanoparticles induced abnormalities in F-actin formation [151]. Exposure of zinc oxide (ZnO) nanoparticles in mouse macrophage cells showed decreased expression of F-actin and its depolymerization [152]. Similar results were observed in human cervical cancer cells (HeLa) and keratinocytes (HaCat) after ZnO nanoparticles treatment [153]. Cells treated with ZnO nanoparticles showed a restructuring of the cytoskeleton. Reorganization of actin and microfilaments into cell bundles was apparent after 2 hours of treatment (Fig. 21.5A) while longer incubation resulted in a spiky style actin organization (Fig. 21.5B) in some cells. However, in other cells polymerized actin was not observed. The most probable mechanism of the effect of ZnO nanoparticles on the cytoskeletal disorganization is given in Fig. 21.5C. Serum protein-bound ZnO nanoparticles interact with cellular receptors on the cell membrane and endocytosed. Endosomes carrying the nanoparticles fuse to lysosomes. The low pH of the endosomal–lysosomal compartment dissolves the ZnO nanoparticles releasing Zn²⁺ inside cytoplasm. Tubulin is a zinc scavenger that undergoes structural changes upon zinc binding and generates abnormal tubulin microtubules. Binding of Zn²⁺ released from ZnO nanoparticles to the actin network might have disturbed the self-assembly of actin microfilaments also [154]. The interaction between TiO₂ nanoparticles and tau proteins showed a reduction in MT density, its disorganization, and disruption of MT [155]. TiO₂ nanoparticles treated MRC-5 lung fibroblasts showed reduced expression of α -actin protein suggesting the possible cytoskeleton remodeling [156]. TiO₂ nanoparticle exposed human lung epithelial cell line (BEAS-2B) indicated the variations in the expression of mRNAs and miRNAs related to the cytoskeletal proteins [157]. SiO₂, TiO₂, and HA nanoparticles affected the MT acetylation and destabilized the MT networks [158]. Improper arrangement of acetylated α -tubulin fibers and β -tubulin was observed on TiO₂ particles exposed cells [159].

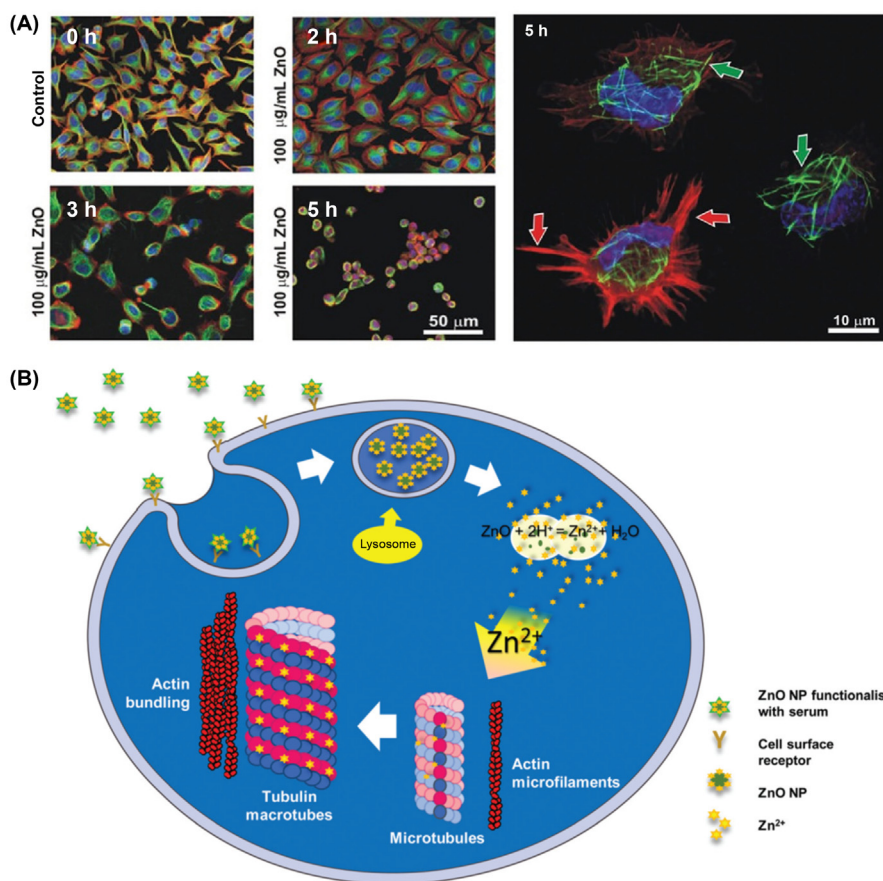


FIGURE 21.5 Interaction between nanoparticles and cytoskeletal proteins. (A) Confocal microscopy image indicating the abnormalities in the structure of the actin microfilaments (red channel) and microtubules (green channel), including F-actin spikes (red arrows) and MT thickening, straightening, and shortening (green arrows) post zinc oxide (ZnO) nanoparticle treatment. (B) Scheme showing the molecular mechanism of the ZnO nanoparticle-mediated cytotoxic effects. MT, Microtubules. Source: Reproduced with permission from García-Hevia L, Valiente R, Martín-Rodríguez R, Renero-Lecuna C, González J, Rodríguez-Fernández L, et al. Nano-ZnO leads to tubulin macrotube assembly and actin bundling, triggering cytoskeletal catastrophe and cell necrosis. *Nanoscale* 2016;8:10963–73. <https://doi.org/10.1039/c6nr00391e>.

Magnetic nanoparticles can also interact with cytoskeletal proteins. Exposure of cells to superparamagnetic iron oxide nanoparticles (SPIONs) has caused considerable decrease in the cell area and polarization due to the F-actin remodeling [160,161]. Disruption of tubulin was also observed on SPION-treated cells [162]. Increased levels MT acetylation was observed in human microvascular endothelial cells upon treatment with SPIONs [163].

Cells exposed to graphene showed significant destabilization of F-actin alignment in pristine graphene-treated cells [138]. However, functionalized graphene-treated cells did not display such adverse effects on cytoskeleton. GO nanosheet-mediated disruption

of intracellular actin filaments was observed in lung carcinoma cells [164]. This could be due to the absorption of actin by GO nanosheets and subsequent changes in the secondary structures of actin monomers. GO nanosheets can cause the separation of the actin tetramer by inserting themselves into the interstrand gap of the tetramer. This can result in the disruption of actin filaments. Similarly, SWCNTs can directly interact with actin proteins and result in filament reorganization [149]. Such interactions mainly happen through hydrophobic interactions. Cells exposed to SWCNTs showed the presence of disorganized tubulin and vinculin [165] that affected the chirality of endothelial cells and morphogenesis. SWCNTs-treated mast cells showed significant actin cytoskeleton disruption and membrane rearrangements [166]. Holt et al. reported that purified and dispersed SWCNTs can induce actin filament bundling in the cells [167]. This resulted in reduced cell proliferation with an increase in actin-related division defects. Loss of cuboidal morphology and the downregulation of tight junction proteins (ZO-1 and occludin) were observed upon treatment with noncytotoxic concentrations of MWCNTs [168]. Such effect was observed after a week of exposure, however, 24 hours of exposure was enough to enhance the tubulin polymerization and fibroblastoid appearance. Actin filament disruption was observed in A549 cells after treatment with graphite CNTs [169]. MWCNTs exposure to HUVECs resulted in the thickening of actin filaments [170]. Such thickened filaments relocated at the cell periphery rather than showing the spreading morphology in the cytoplasm.

Organic-based nanobiomaterials such as liposomes and dendrimers are promising drug delivery systems due to their rapid internalization. Effects of such nanobiomaterials on the cytoskeleton are not well investigated so far. Cationic dendrimer can interact reversibly with actin filaments in a concentration-dependent manner [171]. Actin polymerization was retarded at a low concentration (1 $\mu\text{g/mL}$) of cationic dendrimer whereas at higher concentrations ($\geq 10 \mu\text{g/mL}$), they accelerated actin polymerization. Studies also indicated that uptake of cationic liposomes depends on their interaction with actin networks, whereas endosome-mediated cytoplasmic transport requires the tubulin network [172]. For example, after pulmonary exposure, PEGylated, and aminated dendrimers effectively reached the blood circulation. However, the lung endothelial cells internalized a significantly higher percent of PEGylated dendrimers compared to aminated dendrimers [173]. However, dendrimer-exposed rat hepatocytes showed the extensive colocalization of cytoskeletal protein, zonula occludens-1 with bile salt export pump protein [174].

21.3.4 Nanobiomaterial—organelle interactions

After the internalization of nanobiomaterials, they will be either entrapped within the endosome/lysosome system or free swimming in cytoplasm. An endosome is a membrane-bound organelle of eukaryotic cell that is involved in endocytic intracellular membrane transport pathway originating from the *trans* Golgi network. Nanoparticles uptaken from the cell membrane can follow this pathway to reach lysosomes. Endocytosed nanoparticles have been detected in early endosomes, late endosomes, and lysosomes. Endocytosed nanoparticles are delivered to lysosomes by the direct fusion of late endosomes with lysosomes. Functionalized polystyrene nanoparticles [175] and acidic

PLGA [176] nanoparticles could be internalized by endosomes and transported to lysosomes. Similarly, iron oxide, silica, titanium dioxide, and PLGA nanoparticles were also transported in brain-derived endothelial cells through endosomal/lysosomal pathway [177]. Toxicity of citrate-functionalized silver nanoparticles was mostly dependent on the early endosome formation during clathrin-mediated endocytosis in *Caenorhabditis elegans* [178]. This could be due to the generation of silver ions as a result of the lysosomal dissolution of silver nanoparticles. RAW 264.7 cells which express scavenger receptors also showed endosomal transport and lysosomal dissolution of silver nanoparticles [179]. A major part of internalized silver nanoparticles were localized within endosomal–lysosomal structures rather than in cell nucleus, Golgi complex, or endoplasmic reticulum (ER) of mesenchymal stem cells [180]. Gold nanoparticles can be internalized into cells through endocytosis in a size-dependent manner. Most of the internalized nanoparticles accumulated in lysosomes and resulted in the weakening of lysosomal function by increasing lysosomal pH [181]. However, another study suggested that gold nanoparticles are taken up by the cells through pinocytosis and arranged them in lysosomal bodies in perinuclear fashion without causing adverse effects [182]. GO interacts with other intracellular organelles using the endosome and lysosome as the basement of interactions [183]. In mammalian cells treated with GO and GO nanostructures, these nanostructures were mainly localized in acidic lysosomes [184,185]. The triple negative breast cancer cells (MDA-MB-231) treated with graphene clearly indicated that graphene nanoparticles were located in or around the lysosomes [186]. However, lysosomal membrane permeabilization and cell death was observed when cells were treated with relatively high concentrations of graphene which might be related to lysosome/mitochondria-dependent apoptosis [187]. Phagocytic blood cells (hemocytes) from marine mussels treated with C60-fullerene showed adverse effects on the endocytic-lysosomal system [188]. After 2 days of incubation with PC12 cell, MWCNTs were present in endosomes and lysosomes in an aggregated state [189]. The RNA-wrapped, oxidized double-walled CNTs isolated from cells showed the presence of clathrin-coated vesicles [190]. This indicates that they were sorted in early endosomes, followed by vesicular maturation, engulfed in lysosomes.

Nanomaterials can also interact with mitochondria. Triphenylphosphonium-coupled doxorubicin (DOX)-loaded mesoporous silica nanoparticles have interacted with mitochondrial membrane [191] due to the lipophilic nature and cationic charge. As a positive side of interaction, cerium oxide nanoparticle could effectively inactivate the ROS generated by the defective mitochondria and minimize cell death [192]. Silver and titanium nanoparticles could synergistically act on mitochondria and induce the cytotoxic effect [193]. Exposure of a rat liver cell line (BRL 3A cells) with silver nanoparticles resulted in low GSH levels and an increase in ROS production [194]. Upon exposure to citrate-capped silver nanoparticles, MCF-7 breast cancer cells showed the presence of shorter and more circular mitochondria [195]. This shows the damage of mitochondrial structure upon silver nanoparticle exposure. Moreover, treatment of lung cells with TiO₂ nanoparticles increased mitochondrial ROS production by 46% [196].

The ER is another major cellular organelle with important functions such as proper protein folding and their transport. It is the site for the loading of peptides into MHC class I molecules and the activation of cytotoxic T-cells. Thus, understanding the effect of nanomaterials on the functions and structural integrity of ER is crucial. Exposure to silver

nanoparticles can create ER stress, as evident from the upregulated expression of phosphorylated PKR-like ER kinase (p-PERK), glucose-regulated protein 78 (GRP78), phosphorylated eukaryotic translation initiation factor 2 α (p-eIF2 α), spliced X-box binding protein-1 (XBP1), C/EBP homology protein (CHOP), and phosphorylated inositol-requiring enzyme (p-IRE) [197]. These proteins are involved in the cellular unfolded protein response. Human bronchial epithelial cells (16HBE14o-) exposed to TiO₂ nanoparticles showed the induction of ER stress, disruption of the mitochondria-associated ER membranes, and alteration of calcium ion balance [198]. However, upon treatment with tauroursodeoxycholic acid (an inhibitor of ER stress), alleviated the cellular toxic response. This implies that TiO₂ nanoparticles induced the cytotoxicity on cells through ER stress. ZnO nanoparticles at noncytotoxic concentration can induce endoplasmic reticular stress response as evident from the higher expression of chop, spliced xbp-1, and caspase-12 at the mRNA level, and associated proteins including Chop, BiP, GADD34, p-PERK, p-eIF2 α , and cleaved Caspase-12 at the protein levels [199]. Graphene-based materials also affected the function of ER in cells exposed to them [200]. GO can also regulate ER stress and induce autophagic pathways in nasopharyngeal carcinoma cells [201]. MWCNT exposure promoted the lipid accumulation in THP-1 macrophages and modulated ER stress [202]. MWCNT exposure promoted the expression of ER stress gene DDIT3 as well as ER stress protein p-chop in THP-1 macrophages.

Golgi apparatus is another important cellular organelle where nanobiomaterials can localize and interact. This organelle is important for performing post translational modifications of newly produced proteins. The structural integrity of the Golgi apparatus has a key role in cancer-associated signaling pathways. Inhibiting the Golgi apparatus function can be also be important in certain diseases in which protein misfolding has a role in pathogenesis. In this direction, PLGA–PEG nanoparticles encapsulated with a COX-2 inhibitor (celecoxib) and Brefeldin were developed [203]. Fluorescence microscopic analysis indicated that the nanomaterial containing these two active agents disturbed the Golgi apparatus in murine metastatic breast cancer cells within 30 minutes of treatment. Golgi apparatus-targeting prodrug nanoparticle system based on retinoic acid (RA)-conjugated chondroitin sulfate (CS–RA) accumulated in the Golgi apparatus in cancer cells [204]. This CS–RA exhibited effective inhibition of the expression of several metastasis-associated proteins by the disruption of the Golgi apparatus structure. After injection of gold nanoparticles and electron beam irradiation in tumor bearing mice, accumulation of gold nanoparticles was detected inside the Golgi apparatus of B16F10 cells after 18 hours of incubation [205]. This might have contributed to the higher apoptotic potential of cells upon nanoparticle treatment and irradiation. Graphene-based materials also affected the function of Golgi apparatus in cells exposed to them [200].

21.3.5 Nanobiomaterial–nuclei interactions

Nanomaterials can enter cell nucleus predominantly by two different mechanisms. The first mechanism is by the passive diffusion of free-floating cytoplasmic nanobiomaterials through the nuclear pore complex formation. Generally, the channels will have width of

~6–9 nm [206]. Thus, nanoparticles need to be very small to passively diffuse through nuclear pore complex. Generally, nanoparticles that are smaller than 10 nm can enter the nucleus. The second one is energy-dependent active transport of nanomaterials through the nuclear membrane pore complex. This is achieved by the help of cytoplasmic protein called importins [207]. Nanostructures with diameters up to 50 nm can travel to the cell nucleus by active transport [208]. Tiny gold nanoparticles with 2-nm size transported DNA payloads to the nucleus several folds better than their 14 nm counterparts [206]. Smaller gold nanoparticles modified with PEG and polyarginine (~2.4 nm in diameter) were able to enter in the nucleus while a little larger nanoparticles (5.5–8.2 nm) were circulated in the cytoplasm [209]. Huo et al. investigated the applicability of ultrasmall gold nanoparticles with 2 nm diameter as the carriers for the nuclear delivery of a triplex-forming oligonucleotide (TFO) that binds to the c-myc promoter [210]. Nanoparticle-conjugated TFO was more effective than free TFO to reduce the level of c-myc RNA and c-myc protein expression which indicated the entry of nanoparticle-conjugated TFO into the nucleus (Fig. 21.6). Active transportation of nucleolin-specific aptamers-conjugated gold nanostars to the nucleus resulted in considerable alterations in the nuclear phenotype as evident from nuclear envelope invaginations near the nanomaterial [211]. Such nanoparticle-induced alterations in nuclear phenotype and superior therapeutic efficacy are highly promising for nuclear-targeted cancer therapy.

Some of the nanomaterials entering in the cell nuclei may induce unpredictable genotoxicity due to the small size, surface charge, and other physicochemical properties. For example, gold nanobiomaterials with approximately 1 nm can penetrate into the nucleus and bind to negatively charged DNA due to gold's electronegativity [212,213]. Such DNA binding nanomaterials can create DNA aberrations or totally affect DNA replication. Moreover, ROS and metal ions generated due to the action of nanoparticles such as CuO can damage DNA and affect gene expression [214]. Despite such deleterious effects, drug loaded tiny nanoparticles can penetrate into the nucleus and effectively deliver nuclear-targeted therapeutic agents [210]. Carboxyl derivatized gold-aryl nanoparticles can bind with biodegradable cationic polyelectrolytes such as polydiallyldimethylammonium chloride (PDADMAC). Such gold-aryl nanoparticles treated with PDADMAC can form conjugates with nondisulfide or nonthiol-modified oligonucleotide DNA [215]. Such DNA-nanoconjugate showed resistance against nuclease degradation in the presence of DNase-I.

Semiconductor nanoparticles also show a certain level of interactions with nucleus and harm nucleoproteins and/or chromosomes itself [216]. In addition, oxidative stress induced by the metal oxide nanoparticles can result in nuclear damage. Metal oxide nanoparticles can generate ROS in the cells, mostly in the presence or sometimes in the absence of light, and exert effects on the nucleus [217]. Epidermal growth factor receptor (EGFR)-targeted $\text{Fe}_3\text{O}_4@\text{TiO}_2$ nanoparticles showed higher cellular uptake and nuclear translocation in EGFR-expressing cancer cells (HeLa) [218]. Photoactivation of nuclear-accumulated nanoparticles induced higher double-stranded DNA breaks than photoactivation of nanoparticles that were distributed in the cytoplasm. TiO_2 nanoparticles induced the death of mouse leukemia L1210 cells. In addition to the chromatin condensation, large DNA fragments (2 Mbp) and relatively small DNA fragments (100 kbp to 1 Mbp) were present in cells cultured with TiO_2 nanoparticles. Further degradation of such DNA fragments resulted in the formation of DNA ladders during electrophoresis [219]. Studies also

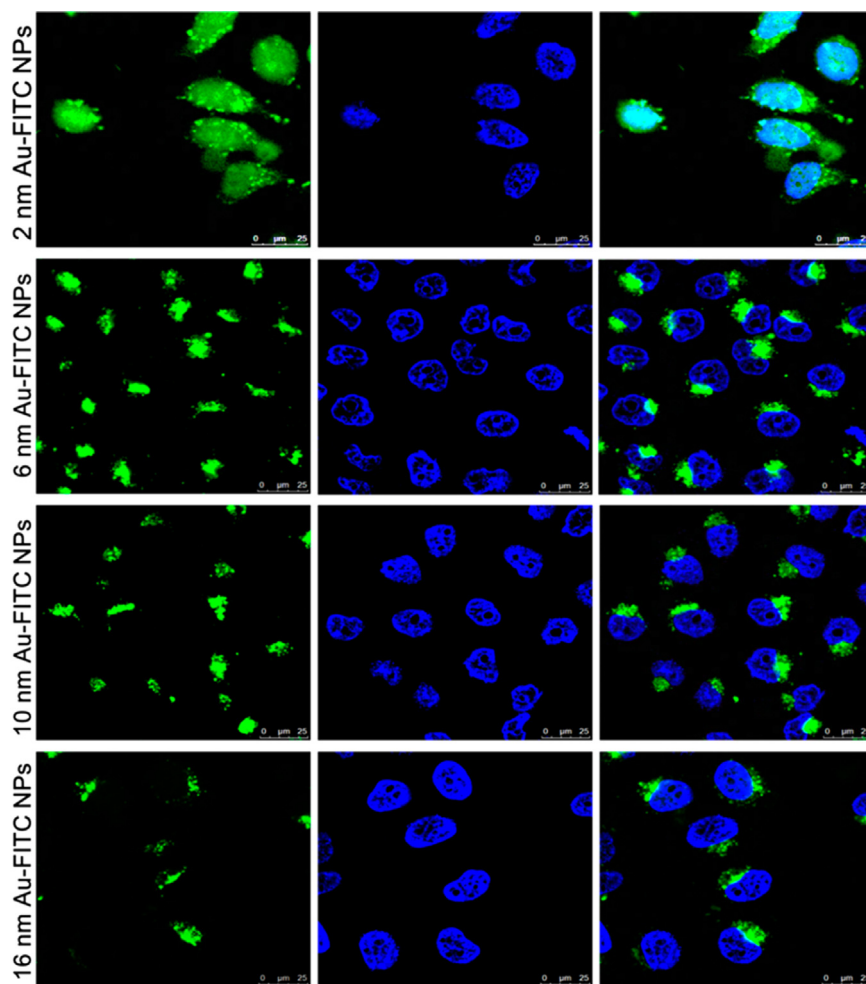


FIGURE 21.6 Intracellular detection of Au-FITC nanoparticles with various sizes in MCF-7 cells. MCF-7S cells were incubated with Au-FITC nanoparticles (green) and nuclei were stained by Hoechst 33342 (blue). Cells were imaged using confocal microscopy. Source: Reproduced with permission from Huo S, Jin S, Ma X, Xue X, Yang K, Kumar A, et al. Ultrasmall gold nanoparticles as carriers for nucleus-based gene therapy due to size-dependent nuclear entry. *ACS Nano* 2014;8:5852–62. <https://doi.org/10.1021/nm5008572>.

showed that ZnO nanoparticles can interact with nuclear components and induce cell cycle arrest at the G2/M checkpoint with visible chromatin changes [220]. Epigenetic changes such as chromatin condensation, increased histone H3K9 methylation and decreased histone H4K5 acetylation were observed in human epidermal keratinocytes. Upregulation of proapoptotic genes such as *Noxa*, *Bax*, and *Puma* and downregulation of *Bcl-xl* was observed in ZnO nanoparticle-treated cells. G2/M cell cycle arrest that happened in ZnO nanoparticle-treated cells might be due to the epigenetic changes and p53-Bax mitochondrial pathway-mediated apoptosis. A significant effect was observed only at

50 $\mu\text{g/mL}$ ZnO nanoparticle concentration. However another study indicated that at 14–20 $\mu\text{g/mL}$ ZnO nanoparticle concentration, DNA damage, increase in the ratio of Bax/Bcl₂, and activation of JNK, p38, and p53^{Ser15} phosphorylation in human liver cells (HepG2) occurred [221].

Carbon-based nanobiomaterials can also interact with the cell's nucleus. For instance, due to the small size, GO-QDs were able to enter the nucleus. Such GO-QDs and nucleus interaction can be used for the nuclear targeting for cancer diagnosis and therapy. For instance, GO-QDs could efficiently deliver anticancer agent DOX to the nucleus through DOX/graphene QDs (GQD) conjugates [222]. This could significantly improve the nuclear delivery of DOX and boost the anticancer potential of DOX. Similarly, *cis*-diaminedichloroplatinum (CDDP) conjugated with GO could enhance the nuclear delivery of CDDP [223].

Studies also shows that edge-functionalization of GQD with N and Cl ligands make them positively charged and amphiphilic for improving the internalization and histone binding in the nuclei [224]. Such nuclei-targeted QDs show multicolor cell imaging potential for the imaging of the ultrafine architectures of the nucleus which is important for nuclear targeting for cancer diagnosis and therapy. Aminated GQD exposed alveolar macrophage cells showed higher cleavage and cross-linking of DNA chains [225]. The dominant forces facilitated the interactions between DNA and aminated GQD were H-bonding and π – π stacking. Moreover, the generation of ROS and the overexpression of caspase genes also might have contributed to the DNA cleavage. N-doped GQD loaded with siRNA can interact with calf thymus DNA (CT-DNA) by intercalative and electrostatic binding [226]. N-doped GQDs successfully cleaved CT-DNA and this indicated its potential to suppress cancer progression. However, an interesting study shows that compared with bare GO and amine functionalized GO, poly(acrylic acid)-functionalized GO induced a less inflammatory response and a little influence on chromatin organization [227]. Such functionalized nanomaterials can be used in drug delivery applications where the drug carriers should be noncytotoxic.

21.4 Conclusion

Nanobiomaterials and their interaction with mammalian cells have been the focus of rigorous research. Generally, nanomaterials can interact with cellular components such as ECM, cell membrane, cytoskeleton, cellular organelle, and nucleus and generate cellular responses. Such responses can be either beneficial or deleterious to the organism. Nanobiomaterial properties such as particle size, shape, surface charge, stiffness, and functionalization which influence the effective penetration through ECM and cell membrane are important factors to be considered when designing nanobiomaterials. A strong interaction between nanomaterials and ECM or cell membranes results in the accumulation of them in ECM or cell membranes and prevent internalization. However, a relatively weak interaction might help to loosely hold the nanomaterials in ECM or cell membrane and facilitate uptake. Similarly, other cellular components also show a diverse range of interactions with various nanomaterials where the extent and outcome of interaction vary from nanomaterial to nanomaterial and cells to cells. However, it is important to thoroughly understand the influence of nanomaterials on cell fate, especially at subcytotoxic

concentrations. Further understanding on the interaction of various nanomaterials with specific cellular components will help to design relatively safe nanoformulations for targeted drug delivery and bioimaging applications.

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SECTION III

Tissue response to biomaterials

Central nervous system responses to biomaterials

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22.1 Introduction

22.1.1 The need for the use of biomaterials in central nervous system

Biomaterials play a critical role in the success of central nervous system (CNS) repair strategies, given their ability to form scaffolds or hydrogel matrices in order to physically support the tissue by bridging the neural gap, guiding the cells or bioactive molecules through the construct with certain mechanical cues and possibly creating sustained delivery systems for migrating cells and/or other nourishing molecules, maximizing the reparative outcome [1–4]. The CNS possesses a severely limited ability to regenerate after an injury occurs due to hostile microenvironment for nerve repair. Some cell replacement does occur after the CNS injury, but the neuronal circuitry is unable to be reinstated [5,6]. Therefore, the challenge for neural tissue engineering is to provide biomaterials that allow for neuronal infiltration and proliferation in the CNS hostile environment without

* The authors contributed equally to this work.

compromising the blood–brain barrier (BBB) or instigating further inflammation. The choice of the correct biomaterial and type of scaffold or hydrogel used are crucial in order to accomplish the desired effect in the targeted tissue. For example, using a hydrogel is preferable for CNS application to achieve: (1) in situ gelation within the CNS lesion; (2) effective retention and stabilization of molecules, cells and/or exosomes used, avoiding rapid clearance and potential generalized adverse effects; (3) mimicking of the CNS micro-environment for effective migration and differentiation of cells and; (4) biodegradability and (5) tunability of the system using appropriate biomaterials as substrates matching the properties (e.g., Young's modulus) of CNS tissue. Biocompatibility is, just like the name suggests, compatibility with the living tissue, and even though this seems important for accomplishing CNS repair, the term is so broad, that testing all aspects related to biocompatibility is challenging and the term is often “abused.” Fig. 22.1 includes aspects that relate to biocompatibility and this makes it obvious that establishing that a material is biocompatible is crucial for the successful clinical translation of a novel therapeutic strategy. The following sections will attempt to provide the necessary focus on the biocompatibility aspects and CNS responses released upon use of each class of biomaterials in an attempt to assist in the clinical translation pathway of neural repair therapeutics [8–11].

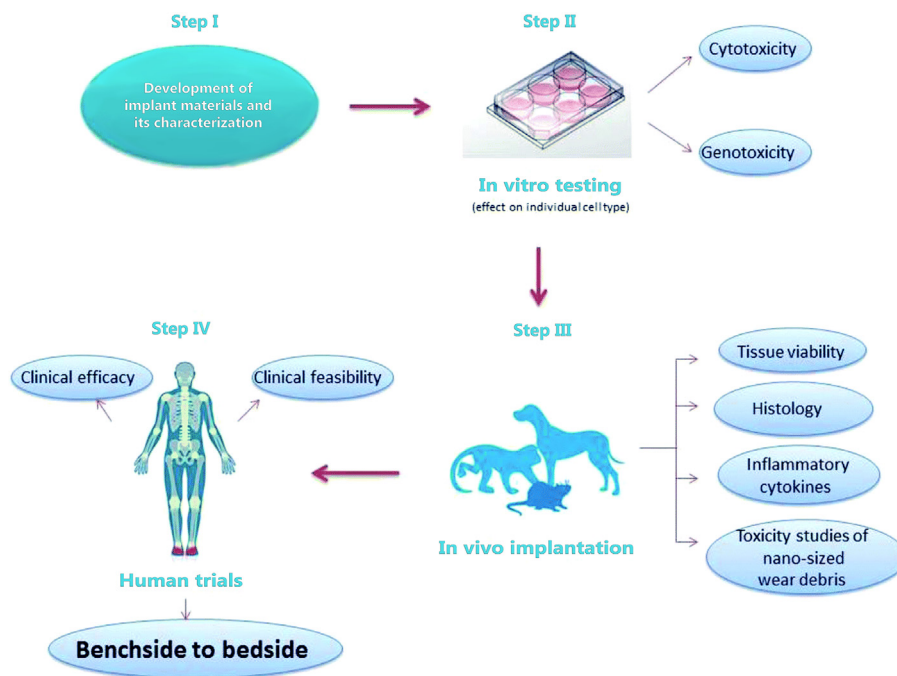


FIGURE 22.1 Biocompatibility importance in the pipeline of clinical translation. Source: Reprinted from Thiruvikraman G, Madras G, Basu B. *In vitro/in vivo* assessment and mechanisms of toxicity of bioceramic materials and its wear particulates. *RSC Adv* 2014;4(25):12763–81; licensed under CC BY-NC 3.0 [7].

22.1.2 Classification of biomaterials used in central nervous system

There are multiple ways that someone could classify the biomaterials used for CNS applications. Based on the required structure and physical and biological properties of prospective tissue construct applied in CNS injury, the biomaterial scaffolds utilized in CNS regeneration can be further classified into hydrogels and biodegradable scaffolds. Nevertheless, this chapter is going to follow a different classification methodology based on the class of the biomaterials used and not based on the structure and mechanical characteristics of the constructs in order to better appreciate the interaction of each class of materials with CNS tissue. Following that classification methodology, the biomaterials for CNS applications can be polymers (synthetic, natural, or conductive), metals, ceramics, or hybrid and composite biomaterials. Sections 22.5.1 and 22.5.2 have been dedicated to certain nanomaterials that are mostly used as part of hybrid/composite biomaterials. Fig. 22.2 provides an illustrative representation of commonly used biomaterials and it gives some insight to potential combinatorial approaches that could establish desired characteristics depending on the targeted field of application. The list of biomaterials discussed in each section below is by no means an exhaustive one, but rather a good representation of commonly used biomaterials in the field of CNS repair.

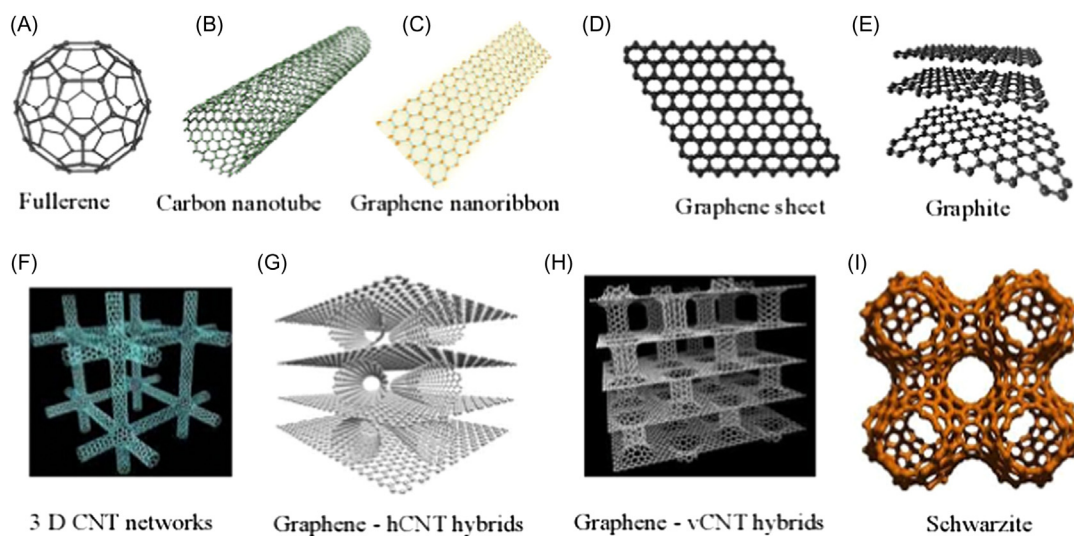


FIGURE 22.2 Combinatorial approaches using biomaterials and nanoparticles for the development of nanocomposite biomaterials. This approach develops new properties for the composite matrix depending on the targeted field of application (e.g., regenerative medicine, drug delivery, etc.). Source: Reprinted from Rafieian S, Mirzadeh H, Mahdavi H, Masoumi ME. A review on nanocomposite hydrogels and their biomedical applications. *Sci Eng Compos Mater* 2019;26(1):154–74; licensed under CC BY 4.0 [12].

22.2 Polymers

22.2.1 Synthetic polymers

Synthetic biomaterials have been extensively used as a substrate for scaffolds or hydrogels for CNS applications. The use of synthetic biomaterials is advantageous due to their mechanical strength and tunability. The mechanical features and degradation rate can be tailored based on the targeted tissue and the immune response can be minimized. Based on certain chemical properties of the polymers the immune reaction can be altered. Generally, hydrophobic materials tend to enhance monocyte adhesion compared to hydrophilic materials, inducing local immune reaction around the biomaterial. Evidence suggests that hydrophilic or neutral biomaterials lead to a decreased monocyte/macrophage adhesion, thereby reducing foreign-body giant cell formation in vitro. On the other hand, adherent cells on hydrophilic or neutral biomaterials have been shown to produce a greater relative level of inflammatory cytokines [13]. In addition, synthetic polymers are compatible with several fabrication techniques like wet-spinning, freeze-drying, electrosin, etc. Nevertheless, there are inherent problems related to the use of such biomaterials. Toxic residual monomers from incomplete polymerization, degradation products, and plasticizers can still be a matter of concern for synthetic polymers, even though they are mostly considered nontoxic. Therefore, it is necessary that synthetic polymers undergo intensive and comprehensive testing prior to clinical translation.

22.2.1.1 *Poly(glycolic acid)/poly(lactic acid)/poly(lactic-co-glycolic acid)*

Poly(glycolic acid) and poly(lactic acid) (PLA) are biodegradable synthetic polymers, which can react to make a copolymer, the poly(lactic-co-glycolic acid) (PLGA) [14–16]. For scaffolds using those materials as a substrate, if hydrolysis of the ester bonds in the backbone of the polymer occurs, then degradation of the scaffold would follow with subsequent release of acidic metabolic by-products that can be absorbed by the host tissue, causing pH decrease around the implantation site. This has also been linked to signs of aseptic inflammation to the implantation site [17,18].

There are several different types of PLA. One example is poly-L-lactic acid (PLLA), a biodegradable synthetic polymer that has been used for CNS applications. Its ultrafine continuous fibers, high surface-to-volume ratio, high porosity, and variable pore-size distribution make it very similar to the extracellular matrix (ECM), while the presence of ester linkages in the polymer's backbone allow for biofunctionalization by covalent conjugation with various molecules. PLLA is considered an ideal substrate for cells for neural tissue engineering applications [19], for example, supporting neural stem cells (NSCs) survival leading to neurite outgrowth in a variety of studies [19,20]. Nevertheless, the polymer is considered to have relatively poor biocompatibility, with insufficient mechanical support during the degradation process and acidic degradation product discharge, making it a less favorable choice [21].

PLGA on the other hand is perhaps one of the most widely studied synthetic copolymers. This is in part due to its approval in drug formulations and medical devices by the Food and Drug Administration (FDA) and partly due to the fact that it is a highly versatile substrate with good biocompatibility, tunable biodegradation rates, and mechanical

properties, as well as the ability to encapsulate many biologically active compounds. This made it an interesting substrate for neural tissue engineering. The deficiency of natural adhesion sites on the polymer has been an area of concern for cell adhesion and growth but several techniques have been explored to promote cell adhesiveness (e.g., hydrolysis, aminolysis, blending, and covalent attachment of adhesive peptides) [22]. With a wide range of options for biofunctionalization of the copolymer and application of different fabrication techniques PLGA is one of the most promising biomaterials for neural tissue engineering, especially when aiming at translational projects. It has been found to reduce cell death, induce neurite ingrowth and, overall, does not cause severe inflammation [23].

22.2.1.2 Poly(ϵ -caprolactone)

Poly(ϵ -caprolactone) (PCL) has been used for CNS applications due to the tunability of degradation rate. Specifically, PCL demonstrates an inherent ability of degradation via hydrolysis of the ester linkages, enabling tunable degradation. It has been used in the form of electrospun nanofiber scaffolds for CNS injuries. For example, in traumatic brain injury (TBI), randomly aligned PCL scaffolds caused significantly reduced microglia and astrocyte activation in Wistar rats compared to the controls, highlighting the lack of significant inflammation that could have arisen from the acidic degradation by-products of the PCL scaffold [24]. PCL, just like PLGA described in Section 22.2.1.1 is found to also reduce cell death, induce neurite ingrowth, without signs of severe inflammation. Nevertheless, PCL possesses a few advantages over PLGA scaffolds, promoting higher levels of neurite ingrowth due to reduced microglia and astrocyte activation [24].

22.2.1.3 Poly(ethylene glycol)/poly(ethylene oxide)

Poly(ethylene glycol) (PEG), also known as poly(ethylene oxide) is a biodegradable synthetic polymer of ethylene oxide units. A particularly attractive characteristic for neural tissue engineering applications is the biomaterial's suitability for hydrogel fabrication due to its hydrophilic properties. It is also highly biocompatible, biochemically inert, and non-immunogenic due to its resistance to protein absorption and cell adhesion. An important aspect to take into account though, is that, contrary to the hydrogels made of natural polymers, PEG-based hydrogels often need to be combined with other polymers because PEG is not bioactive.

Thus, this biomaterial has been extensively used for neural tissue engineering due to its versatile and nontoxic nature, as well as the great potential it showed for treating CNS injuries. Neural cell survival, proliferation, and differentiation have been linked to the use of PEG-based scaffolds for the treatment of CNS injuries [25–27]. Intravenous administration of PEG after severe TBI has reduced neural loss and axonal degeneration to the point that injured and uninjured animals did not demonstrate significant differences [28,29]. PEG has also been shown to seal cell membranes after injury, something highly beneficial to limit cell death. This is also applicable to traumatic spinal cord injury (SCI) where the use of PEG has enhanced the membrane resealing process and accelerated the restoration of mechanical integrity following compression. In addition, Liu et al. used a PLGA/PEG scaffold in a complete transection SCI model in rats, accomplishing enhanced cell growth and migration, as well as improved functional recovery [30].

22.2.1.4 Poly(ethylene-co-vinylacetate)

Poly(ethylene-co-vinyl acetate) (EVA) is not one of the widely used biomaterials for CNS repair. Perhaps its nondegradability has affected its applicability in the CNS. Nevertheless, it is considered a biocompatible polymer, it is also commercially available and has been extensively used for drug delivery applications [31,32]. This might have shifted some of the scientific interest, leading to the biomaterial being recently investigated for neural tissue engineering applications not only in peripheral nervous system (PNS) injuries [33,34], but also in CNS injuries [35].

22.2.1.5 Poly(2-hydroxyethyl methacrylate) and poly(2-hydroxyethyl methacrylate-co-methyl methacrylate)

Similar to EVA, poly(2-hydroxyethyl methacrylate) (pHEMA) and poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) polymers are not degradable, remaining stable after implantation [36–38]. The pHEMA-based scaffold's shape can be manipulated as needed (e.g., channels filled with therapeutics or ECM proteins) [37] and the mechanical properties can be enhanced by the addition of multiple layers of pHEMA [39]. Because of its extremely hydrophilic nature this polymer can easily form hydrogels. pHEMA-based scaffolds or hydrogels are also thought to be biocompatible and can polymerize at low temperatures (-20°C up to $+10^{\circ}\text{C}$), allowing immobilization of functional proteins into hydrogels. The pHEMA-based hydrophilic sponges that were initially developed were enabling axonal regeneration, providing three-dimensional support, while hydrogels using pHEMA as a substrate were able to guide neurite outgrowth [40].

22.2.2 Natural polymers

Natural biomaterials are quite an attractive choice for neural tissue engineering applications given the striking analogies to macromolecular substances of the human body and the similar properties they possess compared to soft tissue they are meant to support, like the neural tissue. They are highly biocompatible for that reason and biodegradable, while chemically tuning their properties is possible. There are even substrates like collagen that are already present in the human body, something that makes the choice for natural biomaterials more attractive, minimizing the risks for cytotoxicity and immunogenic reactions upon the scaffold's implantation [21]. The versatility of natural polymers is also a key for filling and supporting different neuroanatomical locations and adapting for various tissues based on the disorder being targeted [41,42]. The safety profile of natural polymers in neural tissue engineering has been established from the abundance of preclinical work that have even reached the nonhuman primate model without problems. Nevertheless, there are certain limitations that have urged researchers to use caution when favoring natural biomaterials and combining them with synthetic or electroconductive polymers when that choice is appropriate for the tissue. These limitations refer to the weak mechanical properties of the natural polymers, their thermal sensitivity, and certain processing difficulties that frequently necessitate the use of solvents. Overall though, they are excellent choices when used in combination especially for soft tissues like the CNS. Some of those polymers used for CNS applications are described in the subsections below.

22.2.2.1 Agarose/alginate

Agarose and alginate are linear polysaccharides that are obtained from seaweed and algae respectively. Both polymers have been previously used in neural tissue engineering but they must undergo extensive purification in order to prevent immunogenic reactions upon implantation.

Agarose and alginate have been used in the form of hydrogels for CNS applications before [43–48], but they are not found to be adequate for axonal infiltration. In addition, the agarose-based hydrogel's stiffness affects the neurite extension outcomes [43,44]. Agarose still is a good substrate though when it is functionalized with protein gradients (e.g., laminin gradient), promoting and guiding axonal growth up the gradient as suggested by in vitro studies [49]. The agarose-based biomaterials demonstrate good biocompatibility profile and good integration in the host tissue without causing fibrous encapsulation or foreign-body reaction. Immobilization of certain growth factors and bioactive molecules into the channels is possible, enhancing the induced effects of the scaffolds [e.g., immobilization of brain derived neurotrophic factor is shown to promote blood vessel formation with alignment along the longitudinal axis of the channels, while enhancing axonal infiltration into the scaffolds] [50]. Finally, the possibility of tunable in situ gelation is also important for CNS application to establish hydrogel matrices that can conform to the shape of the lesions in situ. For example, in situ gelling of an agarose-based hydrogel system was utilized by Chvatal et al., demonstrating reduction of macrophage infiltration and lesion cavity size upon loading the gels with steroids to treat rodent SCI models [51].

Alginate is also a very attractive choice for neural tissue engineering applications due to its biocompatibility, low toxicity, low cost, and gelation properties. Nevertheless, there are certain drawbacks that warrant caution. The main disadvantage of alginate is the natural presence of impurities (e.g., heavy metals, endotoxins, proteins, polyphenolic compounds, etc.) stemming from its origin. Therefore, a multistep extraction procedure is required in order for the material to be highly purified, minimizing any potential adverse effects, including immunogenic or inflammatory reactions upon implantation of a scaffold/hydrogel [52].

22.2.2.2 Chitosan/methylcellulose/nitrocellulose

Chitosan, methylcellulose, and nitrocellulose are all polysaccharides with similar properties and they have all been used for tissue engineering applications. Cellulose is the most abundant polysaccharide found in nature. Chitosan is a natural, tough, cationic, biodegradable, and biocompatible polymer that is widely used for CNS applications given its capability of enhancing the biocompatibility of synthetic polymers with better mechanical properties and improved reparative outcomes (e.g., enhanced viability and proliferation of PC12 cells) [53–56]. It is the deacetylated version of chitin, which is the second most abundant polysaccharide in nature [57]. When pH is neutral, chitosan forms gels and this has led to the wide acceptance of chitosan-based injectable hydrogels for the CNS tissue. In fact, chitin and chitosan are thought to be ideal biomaterials for biodegradable nerve guides and they are good environments for nerve cell adhesion and neurite outgrowth [58–60]. Functionalization is also possible in order to modify the scaffold based on the scientific needs [61–65].

Methylcellulose on the other hand is also a polymer with great potential for brain regeneration which became a very attractive choice after the approbation of Methocel—a methylcellulose-based system—in nerve repair by the FDA. A reduction in the cavity lesion and glial scar thickness without evidence of inflammatory reaction after methylcellulose-based gels were injected in rodent brains indicated that the biomaterial is biocompatible for brain regeneration [66]. Methylcellulose is able to form thermoresponsive scaffolds, enabling the development of in situ forming injectable hydrogel systems [66,67], something crucial for CNS applications. Nevertheless, methylcellulose gels are not biodegradable, raising problems for neural tissue engineering applications that require biodegradability to avoid adverse effects and scaffold removal surgeries. This limitation can be overcome if methylcellulose gets mixed with other biodegradable polymers like chitosan or hyaluronic acid (HA), developing semibiodegradable gel systems that can be appropriate for brain repair applications [68].

22.2.2.3 Collagen

Collagen is an abundant ECM protein and its presence in the natural microenvironment of the CNS has led to the extensive use and characterization of collagen as a substrate for neural tissue engineering applications. Similarly, gelatin, which is hydrolyzed collagen, has also been widely used for CNS applications. A cross-species transplantation of collagen-based scaffolds can trigger immune response for natural biomaterials like collagen. Humeral immunity is rare for type I collagen, thereby making collagen I a suitable biomaterial for implantation. A simple serological test can verify whether the patient is susceptible to an allergic reaction in response to a collagen-based biomaterial [19]. Cell–collagen interactions occur due to the loci existing on collagen-based scaffolds for the adhesion of cells and the adhesive properties of collagen can be altered by covalent modification [69–71]. The collagen-based scaffolds or gels are also highly tunable. Enhanced collagen concentration for example can increase the cell permeability rate, compressive modulus, cell number, and cell metabolic activity [20]. There have been studies that demonstrated similar physical and mechanical properties of the collagen-based scaffolds to the normal nervous tissue, suggesting excellent biocompatibility profile that can ensure successful neural tissue repair upon implantation [72,73]. Last but not least, the plastic compression of the collagen to form collagen sheet-based conduits has been very useful for PNS applications, but this technique leads to scaffolds that are probably too stiff for CNS repair; therefore if such a technique is utilized for the fabrication of a collagen scaffold, the mechanical properties have to be tuned accordingly in order to match the CNS tissue characteristics as discussed by Tsintou et al. [73].

22.2.2.4 Dextran

Dextran is a complex polysaccharide derived from bacteria and it consists of glucose subunits. Dextran, not only seems to possess antithrombotic properties, but it also forms scaffolds that are resistant to protein and cell adhesion. This is why the dextran-based scaffolds have been investigated for use as coatings for neural implants. In fact, numerous biomedical studies have employed dextran as a biomaterial because it is biocompatible, biodegradable, available in different molecular weights, and it can also be easily derivatized. Functionalization is also possible and dextran can be chemically modified in order

to add selective cell adhesion loci and growth factors [74]. The implementation of different fabrication techniques can also modify the properties of the scaffold to serve its purpose (e.g., certain fabrication techniques allow for microporous dextran scaffolds development that subsequently allow for cellular infiltration) [75].

22.2.2.5 Fibrin/fibronectin

Fibrin is a natural wound healing matrix that forms after an injury and this is the reason why it is such a promising choice for invasive methodologies and tissue engineering applications. Fibrinogen can be obtained from pooled plasma. It can subsequently be cleaved by thrombin into fibrin monomers. A noncovalent scaffold can be formed from the assembly of those monomers and it can offer sites for cell adhesion [76]. Covalent modification of fibrin also alters its properties [77–80].

Fibronectin is a high molecular weight glycoprotein, which can be obtained from bovine or human plasma and can bind collagen, fibrin, and heparin. Its soluble form is found in the blood and it serves a role in the wound healing process. Given its capability of aggregating to form mats, it has been used as a scaffold for neural tissue repair [81–85]. The pores existing in the mats are great for providing guidance of the regenerating neurons towards the same direction, something that can be important especially for larger lesions like the ones found in primates. They also provide cell adhesion sites and they can absorb growth factors, acting as a reservoir.

Different types of stem cells have been encapsulated in fibrin gels successfully. In a sub-acute rat model of SCI for example pluripotent stem cells and growth factors [e.g., neurotrophin-3 (NT-3) and platelet-derived growth factor] were embedded in a fibrin gel with demonstration of an increased survival of the transplanted cells, resulting in the differentiation of more neurons [86]. Despite the promising outcomes accomplished with the use of fibrin, this biomaterial alone is not a top choice for CNS applications because of its fast degradation. The combination of fibronectin with fibrinogen has been attempted in order to resolve this issue and increase durability, maximizing efficiency of those gels [87]. Indeed, the fibronectin/fibrinogen gels have demonstrated superior properties, being more suitable for SCI regeneration application, compared to each biomaterial alone or even compared to collagen gels. Good integration with the host tissue, axonal growth with Schwann cells infiltration and laminin deposition, abundant vascularization, and nonformation of cavities have been some of the observed outcomes in the nervous tissue.

22.2.2.6 Hyaluronan/hyaluronic acid

HA or hyaluronan is a linear glycosaminoglycan also found in the ECM of the brain, something that makes it an attractive choice for CNS tissue engineering purposes. It is a very attractive biomaterial because of its tunability in terms of biodegradability, biocompatibility, bioresorbability, and hydrogel forming ability [88]. Nevertheless, HA does not promote axonal attachment and growth, impeding neuroregeneration. Reduction of the lesion cavity, as well as reduction of the inflammation and gliotic scar in the surrounding tissue is noted though. The decrease in the inflammatory response can be justified considering the negative charge of the HA chain that inhibits cell attachment and binding interaction with the cell membrane receptor CD44 of macrophages [89]. The high biocompatibility of HA with reduction of the inflammatory response has been invaluable for minimizing the

inflammatory reaction induced by electroconductive polymers in neural tissue engineering applications. For example, it was suggested that pyrrole/HA conjugates could potentially mask conducting electrodes from postimplantation adverse glial response [90]. HA hydrogels hold great promise for therapeutic interventions to the CNS given their ability to enhance the survival rates and proliferation of neural precursor cells [91–93]. Its widespread application for neural tissue engineering projects is probably related to the fact that this biomaterial has demonstrated on a variety of substrates neurite outgrowth, differentiation, and proliferation. By influencing the differentiation of neural progenitor cells due to certain mechanical properties, HA-based hydrogels show promise for targeting neurodegenerative conditions [94,95]. One drawback of using HA for neural tissue engineering applications is that HA is water soluble, thereafter making it hard to develop injectable formulations unless additional components (e.g., cross-linkers) are included to stabilize the scaffold [72,96,97].

22.2.3 Conductive polymers

Electrical stimulation has been shown to promote the processes involved in neuroregeneration so the application of electrically conductive polymers, which are normally polymers with loosely held electrons along their backbones, is a very promising approach. The main conductive polymers discussed below are the most widely used biomaterials, such as polypyrrole (PPy), polyaniline (PANI), poly(3,4-ethylenedioxythiophene) (PEDOT), graphene, and carbon nanotubes (CNTs) [98,99], even though CNTs and graphene are analyzed in detail in the [Section 22.5.2](#) among other carbon nanomaterials. The main reason why those materials have attracted attention for neural tissue engineering purposes is the biomaterials' tunability in terms of a variety of properties such as stability, electrical conductivity, and ability to act as a delivery system for biomolecules. Depending on the specific application, their electrical, chemical, and physical properties can be modified accordingly. In order to accomplish purposeful manipulation of the electrical properties of conductive materials, the biomaterials have to undergo a process called doping. This process usually includes adding chemical reactants to oxidize or reduce the systems in order for electrons to be pushed into the conductive orbital within the potentially conductive system. Nevertheless, functionalization with the process of doping can affect the conductivity of the resulting materials [100,101].

Overall, despite the promise that intrinsic conductive polymers hold for neuroregeneration applications, there is a critical issue in their use, namely their suboptimal biocompatibility, associated with their inability to degrade *in vivo*, which could thereafter induce chronic inflammatory reactions and immunogenicity, requiring additional treatments, including invasive manipulations (e.g., additional surgeries) [102]. Other issues linked to the use of intrinsic conductive polymers include the poor polymer–cell interactions, the absence of cell interaction sites, hydrophobicity, processability, and mechanical properties [103]. In an attempt to overcome this issue, it has become a common practice for conductive polymers to be blended with other biodegradable polymers, both synthetic and natural, resulting in systems with crucial electroconductive properties into more biologically favorable biomaterials. PLA, PCL, PLGA, polyurethane, chitosan, gelatin, and collagen are

only some of the biomaterials used for developing desirable composites aiming at biodegradation improvements. Another way to overcome the limitations related to the use of such biomaterials is by using biofunctionalization techniques for synthesizing erodible conducting polymers [104] or for preparing degradable conductive polymers containing conducting oligomers [103]. In regard to electroactive polymer composites, one major drawback linked to the use of carbon nanomaterials is the potential toxicity [105]. It should be noted that even though carbon nanomaterials are not biodegradable, they are still being excreted *in vivo* to accomplish body clearance.

Caution should be exercised with more studies exploring any potential cytotoxicity due to the long-term electrical exposure of cells [106]. Mechanistically speaking, it is speculated that reduction of the intrinsic conductive polymer (e.g., PPy and the electric conduction itself can both affect cells in numerous ways). One example is the process of neutralization of PPy, under a reducing potential, which results in the expulsion of negative ions or the uptake of positive ions, such as Na^+ from the medium, potentially affecting several processes, including protein adsorption and the cell cycle. For instance, when comparing electrically stimulated and nonstimulated composites of PPy/chitosan with embedded human adipose-derived mesenchymal stem cells, stimulation under direct current for 7 days led to a calcium deposition 346% higher in the stimulated scaffolds [107].

22.2.3.1 Polypyrrole

PPy is an organic polymer formed by the polymerization of pyrrole monomer and it is one of the most widely used conductive polymers in neural tissue engineering, with applications in many fields such as drug delivery, nerve regeneration, and biosensor coatings for neural probes applications [108]. This biomaterial demonstrates a relatively good (but still suboptimal) biocompatibility and it can support cell adhesion and growth of a number of diverse cell types, making PPy a great candidate for tissue engineering. Nevertheless, this biomaterial is very hard to be applied alone, but it has to be changed into a mechanically manageable and processable material first. This is because conjugation in the molecular backbone of PPy leads to its rigidity, insolubility, and poor process ability [98].

In order to enhance biocompatibility, PPy is mainly used in combination with other biodegradable unnatural polymers such as PLA, PLGA, and PCL. Some examples include PPy-coated PLGA electrospun nanofibers, which led to an increased neurite growth and differentiation, combining the effects of electrostimulation and topographical guidance [109,110], PPy–PLA fibers, which demonstrated enhanced neurite adhesion, alignment, and elongation [111], and PPy–PCL films, which supported cell proliferation and enhanced neurite outgrowth through electrical stimulation both *in vitro* and *in vivo* [112]. These biomaterials are also combined with natural polymers, such as HA in an attempt to construct three-dimensional electroconductive hydrogels for improving recovery post TBI and stroke [113,114].

A very interesting application of PPy regards its use as a new electrode material for long-term chronically implantable neuroprosthetic devices [115,116]. In an attempt to limit adverse immunological reactions and improving direct tissue integration, PPy has been immersed into plasma with promising results. This technique has been used by Kondyurin et al. in order to develop a biologically active electrostimulating neural

interface [117]. Controlled localized drug delivery to the CNS has also been accomplished with the use of PPy as an electrically controlled vehicle for the release [118–120].

22.2.3.2 Polyaniline

PANi is another conductive polymer that is in the list of the most widely used ones due to the many attractive properties it possesses (e.g., high conductivity, easy synthesis, low cost, and easy availability) [121]. In order to mitigate inflammatory and immunogenic reactions, tackling the problem of suboptimal biocompatibility, like with PPy, this biomaterial is often combined with more suitable biodegradable polymers (e.g., PLA–PCL) [122] for neural tissue engineering applications. Even though it has been designed as a hydrogel for neural tissue engineering with great promise for peripheral nerve regeneration, it has also been used as a substrate for NSCs differentiation [123,124] and more recently it has showed promise as a bio-sensing electronic patch, which could get integrated in electroresponsive tissues for recording and therapeutic stimulation, thereafter bringing up more research potential in this field [125].

22.2.3.3 Poly(3,4-ethylenedioxythiophene)

PEDOT is a very interesting choice within the electroconductive polymers with multiple applications in neural tissue engineering, bearing optical transparency in its conductive state, high stability, and low redox potential. This biomaterial has mostly been used for microelectrodes aimed at neural electrical stimulation and recording [126–128]. One indicative example comes from Cui's research group, which has developed numerous PEDOT-based electrodes for stable neural recording and therapeutic stimulation [129–131]. More recently, PEDOT has also been used for NSC differentiation through electrical stimulation, resulting in longer neurite outgrowth and longer neurons [132,133].

22.2.3.4 Indium phosphide

InP is a direct band semiconductor, which is usually utilized for superior optoelectronic interfaces, and even though it is not one of the most widely used conductive biomaterials for neural tissue engineering applications, it has shown promise, bearing a potential for the development of nanoscale tissue engineering constructs that could also be used for sensing and stimulating cellular activity in the brain. InP nanowires in particular have recently been shown to influence hippocampal and cortical cellular growth in rodents, neuronal and cell morphology, circuit formation and function, utilizing nanoscale topographical features [134]. In the same study InP nanowires have been demonstrated to be noncytotoxic, while the InP nanowire patterns have been shown to be an effective physical cue to induce neurite growth on two-dimensional (2D) substrates, with neurite guiding occurring via mechanosensing. This was the first study that has studied the biocompatibility and toxicity of the material with the results being positive for the neuronal cells.

22.2.3.5 Carbon nanomaterials (i.e., graphene, carbon nanotubes)

Graphene and CNTs are two kinds of biomaterials that belong in the carbon nanomaterials category, but are also used for their electroconductivity.

Graphene has been called a miracle biomaterial for a good reason; it is an exceptionally good conductor of electricity and heat and is very strong, flexible, and stable. In the world of biomaterials, graphene is a great candidate due to its excellent mechanical and electric

properties, as well as due to its biocompatibility with human cells [135,136]. It is frequently used to provide mechanical support to hydrogels that need strengthening, as well as an electric filler for percolated conductivity polymers [137].

CNTs are other types of conducting polymers which have electrical conductivity. CNTs can be functionalized based on the application; one of the ways to functionalize CNTs includes substitution reactions, such as replacement of carbon atoms from the tube wall by boron or nitrogen [98]. The functionalized CNTs have been shown to be useful for promoting neural signal transport and help dendrite elongation and cell adhesion [98]. For example, neuronal growth and neurite elongation have been observed in all directions in a study where dissociated embryonic rat hippocampal neurons on a mat of multiwalled CNTs (MWCNTs) were deposited onto a polyethyleneimine (PEI) covered glass [138]. When combined to create a composite scaffold to optimize the properties for neural tissue engineering applications, promising results have been yielded under electrostimulation, enhancing proliferation, differentiation, and growth of long neurites [139]. For example, in this study where polyacrylonitrile/carbon nanofibers (CNFs) composites were developed, NSCs got proliferated, demonstrating improved differentiation and maturation under 5 V [alternating current (AC)] for 4 hours during 7 days [140]. In another study a different combination of materials, namely PLGA/CNT, was used to build other electroconductive composite scaffolds in order to test the difference between stimulated and nonstimulated cells under an electric current (AC), demonstrating improved outcomes for the stimulated nerve cells in terms of cell proliferation, differentiation, neurite outgrowth, and myelination [139].

A more detailed analysis of the whole category of carbon nanomaterials is included in Section 22.5.2.

22.3 Metals

22.3.1 Introduction and unspecific toxicities

Metal grafting in the CNS is based upon chromium and cobalt or nickel and titanium (nitinol) alloys that may contain tungsten or iridium components as well as stainless steel or platinum. Alloys with these elements are used for implants that are most frequently applied rather in vascular diseases as stents, flow-diverters, or coils but also as materials for deep brain stimulators and electrodes, for example, in epilepsy diagnostics.

The alloy unspecific adverse effects include ischemia as a result of clotting due to vascular location and implant infections, electrical stimulation, and oxidizing processes. Exogenous electrical stimulation itself is a noxa, independent from the electrode material, leading to cellular membrane disruptions and vacuolation that in turn enhance the vulnerability to element specific toxicities [141]. Further, the oxidative potential results from iron, copper, titanium, chromium, and cobalt. With solely four electron shells and being classical substrates of the Fenton or Fenton-like reactions, these elements induce oxidative stress and lead to reduced neural viability.

Most metals mentioned below seem to interfere with hippocampal homeostasis leading to increased vulnerability, as the dentate gyrus is one of the few areas with neuronal stem cells.

Nevertheless, the adverse effects of metal grafting in the CNS are limited to neighboring tissue due to irrelevant systemic fluctuations in metals' concentrations in comparison to

hip replacement surgeries, having the potential to increase serum metal levels and leading to altered neural metabolism. Finally, metals' neurocompatibility relies additionally upon the material surface and coating [142].

22.3.2 Iron (Fe)

Regarding specific effects, direct neurotoxicity and indirect oxidizing effects of iron make this element less frequently used in direct contact with nervous system despite its almost ubiquitary presence. Iron uptake begins within short period after the onset of stimulation or exposure and decreases the neural viability [143]. Therefore, iron alloys are rather used in vascular stenting thus reducing the direct contact to the neural tissue.

22.3.3 Chromium (Cr)

Chromium is an essential element and as Cr(III)-ions cofactor in various enzymes in the human body. However, Cr(VI) complexes are known for their carcinogenic potency. Chromium is a strong oxidant agent leading not only to a cytoplasmic Fenton-like reaction but also interfering with physiological mitochondrial metabolism inducing apoptosis and disrupting the local astrocyte homeostasis and therefore inducing classical gliosis and astrocytic expression of factors damaging the neurons [144].

22.3.4 Cobalt (Co)

Neurotoxicity observed in cobalt relies upon similar mechanisms to chromium, such as induction of caspases by interacting with mitochondrial metabolism [145,146]. Further, cobalt nanoparticles (NPs) were shown to create damage especially to the hippocampus and the temporal cortex [147].

22.3.5 Molybdenum (Mo)

Molybdenum frequently added to chromium and cobalt alloys and mostly used in orthopedic surgery, for example, in spinal applications, increases the amount of free metals during the slow material deterioration subsequently causing production of reactive oxygen species and secondary metabolism changes in neural cells [148]. The second, indirect damaging mechanism is the altered astrocytic microenvironment causing neural degeneration.

22.3.6 Nickel (Ni)

In nickel and titanium alloys (nitinol), the neural uptake of nickel nanoparticles may cause, beside the oxidation mentioned above, concentration-dependent DNA damage and cell death [149]. The observed induction of caspases contributes to aging and degeneration processes such as synucleinopathies [150]. Nevertheless, nickel remains less toxic in comparison to other metals, despite being known for inducing contact allergic reactions [151].

22.3.7 Titanium (Ti)

Titanium alloy toxicities include increased apoptosis rate, particularly in the hippocampus, as well as a decrease in neuronal maturation, which may rely not only upon the oxidative stress but also upon fluctuations of intracellular calcium levels [152,153].

22.3.8 Tungsten (W) and iridium (Ir)

Both tungsten and iridium are used in alloys for coils, stents, and implantable stimulator electrodes. Tungsten itself was shown to create oxidative stress, even though it does not belong to the group mentioned above, and thus altering local, especially hippocampal, neurotransmitting systems by interacting with potassium-gated channels [154,155]. Iridium remains not well researched regarding its influence on neural viability.

22.3.9 Platinum (Pt)

Platinum is known as direct noxa for the neurons in chemotherapy causing syndrome of inappropriate secretion of antidiuretic hormone or encephalopathies [156]. Neurotoxicities following local applications in the CNS remain not sufficiently studied.

22.3.10 Management of metal induced toxicities

The management of toxicities in the CNS is limited, especially in local applications of small implants. A discussed substance that may prevent oxidation-related deterioration is acetyl cysteine, however, without a sufficient degree of evidence and rather in experimental approaches [154]. In various metal toxicities, chelation is seen as an approach for causal treatment. Chelates used in metal toxicities are sodium-diethyldicarbamate for nickel and ethylenediaminetetraacetic acid for iron [157]. Inflammatory and intolerance reactions are manageable solely by graft removal.

22.4 Ceramics

Ceramic is a wide group of materials including oxides of silicon, aluminum, and titanium and or other inorganic formations. Its field of application includes microelectrode coating as well as prosthetics of the vertebral column.

22.4.1 Silicon oxides

The direct toxicity of silicon-derived ceramics on CNS remains not well investigated. There are hypotheses based upon observations in animal models that silica can alter the brain-blood barrier, as well as induce oxidative processes [158,159].

22.4.2 Aluminum oxides

Al_2O_3 a common compound shows interactions with local serotonergic, dopaminergic, and glutamatergic transmitter in animal models [160]. Coadministration of aluminum materials and medicine or implants containing platinum leads to interaction in the form of toxic precipitates with low solubility [156]. Aluminum was also associated with dialysis-induced central nervous toxicity, which leads to severe dementia and other focal neurological deficits in various patients [157].

22.4.3 Titanium oxides

In in vitro experiments titanium dioxide contributes to neurotoxicity of dopaminergic cell models by causing oxidative stress, increasing the apoptosis rate by activating the c-Jun n-terminal kinase, increasing the fibrillation of α -synuclein, and causing local raising lactate dehydrogenase levels in neural cell cultures [161,162]. Regarding epidural implants, the toxicity is restricted to epidural fibrosis without causing near CNS inflammation or pathology [163]. Adverse effects of the entire CNS such as cognitive decline or neuro-ocular side effects can occur if the blood concentration of cobalt, titanium, or aluminum ions rises noticeably, for example, after implantation of a large device such as a prosthesis or oral intake [164–166].

The overall toxicity of ceramics remains not well investigated and hypothetically as well as practically low. Direct trauma and astrocytic reactions leading to glia scar are the most common reactions and the reason for deterioration in implant function.

22.5 Hybrid or composite biomaterials

Hybrid or composite biomaterials are engineered biomaterials derived by the combination of multiple biomaterials in an attempt to produce a superior matrix for the intended application. This need has arisen due to the fact that there is no perfect biomaterial to accomplish the desired cellular and tissue behavior. The combination of natural and synthetic polymers with numerous other biomaterials has demonstrated the ability to enhance cellular interaction, encourage integration into host tissue, and provide tunable material properties and degradation kinetics.

A major problem that needs to be tackled after CNS damage is the development of a cavitary lesion. Such cavities are often a result of TBI, late phase stroke remodeling, or certain neurodegenerative diseases and they inhibit nerve regeneration potentials. This is why structural mechanical support to bridge the lesion for the neurons to have an appropriate substrate for guidance, growth, and elongation is very important. The research on this topic has been focused on the development of gels in order for the material to conform to the lesion's shape and size and allow in situ injection of the matrices [61,167,168]. Composite hydrogels can be tuned as needed in order to provide the necessary control for the optimal CNS regeneration to be accomplished.

There are several studies focused on developing and testing composite hydrogels with very promising results. For example, when HA hydrogels were compared to HA-laminin

composite hydrogels, both types of hydrogels led to glial scar reduction, increased integration into the surrounding parenchyma, increased cell infiltration and increased angiogenesis, but neurite migration, extension, and regrowth were only observed in the composite hydrogels [169]. In another study, coencapsulation of biomolecules and cells was tested in an attempt to enhance the efficiency of the hydrogel system in terms of neural repair-related processes. In particular, collagen, basic fibroblast growth factor-2, and neural precursor cells were coencapsulated in a composite PEG/PLA hydrogel, modifying the microenvironment surrounding the cells within the three-dimensional composite hydrogel in such a way that neural cell survival and metabolic activity were increased [69]. In addition, composite hydrogel systems can be combined with a delivery system with different properties and release profile (e.g., nano- or microspheres with another encapsulated biomolecule) to accomplish tunable independent release of certain factors in different time points depending on the engineering of the composite matrix for the intended application. One example is the use of PEG/PLA composite hydrogels with embedded ciliary neurotrophic factor, but also with PLGA microspheres that have NT-3 encapsulated. Such a system allows distinct release kinetics for the delivery of neurotrophins and this way the hydrogel system can be optimized and tuned in a way that allows the microenvironment to be altered following a more physiological temporal sequence that would in turn maximize the regenerative potential [170].

Another biomaterial that has been investigated as part of composite scaffolds is single-walled CNTs (SWCNTs). This is because of their high mechanical stability, corrosion resistance, and electrical conductivity [171,172]. For example, increased cell viability of NG108-15 neuroblastoma and glioma hybrid cultured cells was accomplished in composite films comprised of poly(diallyldimethylammonium chloride) (PDAA) and layer-by-layer (LBL) assembly of SWCNTs and poly(acrylic acid) (PAA) compared to PDAA or PAA films alone [173]. In addition, in another study the use of SWCNTs for developing composite matrices explored potential issues in terms of biocompatibility. Nevertheless, the LBL films of PEI and SWCNTs demonstrated no adverse effects on the viability and differentiation of NSCs. Therefore, this could potentially be an appropriate choice for neural prosthetic devices based on this study [174]. Another study took this one step further, exploring not only biocompatibility, but also potential improvements of the system in terms of nerve repair due to the addition of SWCNTs. This study in fact showed that electroconductive LBL PAA/SWCNT thin films were not only biocompatible, but they could also induce an electrophysiological response from differentiated NG108-15 cells in vitro [175]. These in vitro studies suggested that SWCNTs are not only an appropriate and biocompatible choice for neural tissue engineering applications as part of a composite system, but they could also potentially stimulate cells to regain neural functionality when implanted as devices for neural regeneration.

In terms of SCI, this disease is considered an orphan disease contrary to other CNS conditions, affecting fewer than 200,000 people nationwide, based on the FDA definition. This has made the discovery of SCI-targeted therapeutic strategies even more challenging, with the research progress in this field following a much slower pace compared to the progress on therapies for other CNS conditions (e.g., stroke). So far, no treatment has been effective to be established as the standard of care for SCI patients, even though numerous single natural (collagen, alginate, HA) and synthetic polymers (PEG, PLGA, polycarbonate) have been used to manufacture gels, sponges, and tubes for neural tract regeneration in

SCI [176,177]. Compared to the approaches explored for other CNS conditions, composite matrices for SCI applications gained attention later. The results were very promising on utilizing such techniques in order to engineer a composite that would be more effective for SCI axonal regeneration. For example, copper-capillary alginate gels with a linear microtubular structure have been complexed with oligochitosan to prevent dissolution [178], demonstrating biocompatibility with mouse embryonic stem cells and capability of inducing long cylindrical cellular structures within the microtubules.

All those studies stress the potentially beneficial role of the use of combinatorial approaches towards neuronal and neuroglia regrowth. Nevertheless, the higher level of control and tunability of composite matrices comes with the cost of having to explore the optimal settings for a variety of parameters. Therefore, more research is required in order to determine the optimal cell source, the role of inflammatory factors on these constructs, and their mechanical properties. The microenvironment should also be taken into account. For example, a hypoxic microenvironment is common after CNS injury, therefore the regenerative effects of constructs developed for neural engineering applications postinjury should be assessed for different oxygen concentration levels.

22.5.1 Interaction of nanomaterials and nanoparticles with central nervous system

In an attempt to exploit the beneficial properties of nanomaterials (e.g., the increased surface area to volume ratio), nanomaterials have become very attractive candidates in biomedical science, including diagnosis, drug delivery, and development of human organs. Several nanomaterials have already been studied even as part of clinical trials and not only as part of preclinical studies.

Especially for CNS applications, the use of NPs is of particular importance due to the presence of the BBB which normally, when intact, limits entry of proinflammatory and neurotoxic blood-derived factors into the brain parenchyma. The fact that only small molecules that are lipid soluble with a molecular weight <400 Da can cross the BBB (most macromolecules are unable to penetrate the brain endothelium), hinders CNS drug development. This is why the use of NPs is on the one hand revolutionary and beneficial to accomplish biomolecule delivery which was not possible before for therapeutic purposes, but on the other hand it can be problematic with potential toxic effects. Of course, the BBB is one of the pathways allowing the NPs access to the brain. Other major pathways include the nose to brain pathway and the placental barrier, with each of which present unique components [179].

The interaction of the CNS tissue with NPs is therefore different than its interaction with other biomaterials that are out of the nanoscale. Depending on the NPs' physico-chemical properties, they result in a variety of neurotoxic mechanisms after brain penetration. Upon interaction of the NPs with the local cells standing for the CNS immune functions, a toxic reaction gets initiated including oxidative stress, apoptosis, and other inflammatory pathways. The oxidative stress and subsequent mitochondrial damage can in turn lead to aggregation of protein strands and nonspecific posttranslational modifications, just like observed in CNS pathologies like Alzheimer's disease or Parkinson's

disease (PD). NPs are also able to act as modifiers of cellular autophagic response, resulting in neurodegeneration. The toxic profile of the NPs depends on properties like the NP size, shape, zeta potential, as well as the aggregation and dispersion status.

For example, the tiny size range of NPs increases the potential of interaction with nucleic acids, proteins, and other biomolecules or certain organs like lungs. To understand how tiny NPs are, Seeman [180] compared the size of NPs with red blood cells (RBCs) with the NPs being 100 times smaller than that of RBCs. NPs, which are well known for their potent neurotoxic effects, include SWCNTs and MWCNTs, metal oxides like silicon oxide (SiO_x), titanium dioxide (TiO_2), etc.; such NPs act like neurotoxins for CNS and PNS [181]. In addition, fullerenes, which are one type of nanomaterial belonging to the large category of carbon nanomaterials, were found to induce oxidative stress in the brain of a largemouth bass in juvenile stage [182].

In summary, even though NPs have attracted a lot of attention in recent years with extensive research work on the subject, still the precise toxic effects of NPs along with the potential underlying mechanisms involved have not been elucidated in detail. Understanding those mechanisms, as well as the role of NPs in originating neurological conditions could potentially allow the circumvention of neurotoxicity and exploitation of the positive characteristics alone for the development of novel therapeutic strategies. Thus, it is highly significant for future research to be directed to the involved molecular mechanisms of neurotoxicity related to the use of NPs.

22.5.2 Carbon nanomaterials

Carbon-based nanomaterials present exclusive electrical, mechanical, and biological characteristics with low toxicity, high mechanical strength, high thermal, and electric conductivity, as well as, in some cases, intrinsic fluorescence. These nanomaterials can interface with cerebral tissues, and they can be used for imaging applications, neuronal growth, neuroprotection, and drug delivery tasks with high efficiency. A reference to the wide variety of carbon nanomaterials for numerous CNS applications is offered in Table 22.1. In general, those characteristics place carbon nanomaterials in a unique place for tissue engineering applications. Carbon allotropes with a particular focus on the carbon nanoforms are illustrated in Fig. 22.3 for the sake of visualization. In particular, graphene and CNTs are the main nanomaterials utilized for neural tissue engineering applications due to their electroconductivity, flexibility, and biocompatibility. Nevertheless, the understanding of the biological interactions of carbon nanomaterials in vivo is limited. Hence, further investigation is needed in order to explore their in vivo interactions with singular cellular and immunological components, as well as in order to map the ultimate cycle (e.g., accumulation, degradation, and/or excretion) of those nanomaterials in vivo.

22.5.2.1 Carbon nanotubes

CNTs are carbon nanomaterials that consist of continuous rolled-up graphitic foils. Depending on the number of graphitic tubes, they can either be categorized as SWCNTs, if consisting of a single graphitic tube, or categorized as multiwalled (MWCNTs), if more concentric tubes are present. Their diameter ranges from 0.7 to 5 nm for SWCNTs and

TABLE 22.1 Carbon nanomaterials used for central nervous system applications.

Carbon nanomaterial	Chemical modifications	Application	Animal model	Administration route and dose	In vivo toxicity	References
SWCNTs	^{13}C enriched + Tween 80 (1%)	Biodistribution analysis	Mouse	i.v. 200 μg	Moderate (lungs, liver)	Yang et al. [183]
	$-\text{NH}_2$	Neuroprotection	MCAO stroke model rat	i.c.v. 40 ng	No	Lee et al. [184]
	PEG	Neuroregeneration	Spinal cord injury model rat	Lesion site 0.025–2.5 μg	No	Roman et al. [185]
	PEG	Toxicity analysis	Rat	i.cr. 0.5–1.0 μg	Yes	Dal Bosco et al. [186]
	Acetylcholine	Drug delivery	Mouse	g.i. 25 mg/mg	n.a.	Yang et al. [259]
	PEG oligonucleotide (CpG)	Drug delivery	Glioma-bearing mouse	i.cr. 7.5 μg	No	Zhao et al. [187]
MWCNTs	PF-127 coating	Toxicity assessment	Mouse	i.cr. 30–150 ng	No	Bardi et al. [188]
	^{111}In]DTPA	BBB crossing analysis	Mouse	i.v. 50 μg	n.a.	Kafa et al. [189]
	$-\text{NH}_3^+$ + siRNA	Neuroprotection	Endothelyn-1 stroke model rat	i.cr. 0.5 μg	No	Al-Jamal et al. [186]
	Oxidation PEG doxorubicin angiopep-2	Targeted drug delivery	Glioma-bearing mouse	i.v. 1.9 and 6.3 mg/kg	Cardiac, lower than DOX	Ren et al. [191]
	$-\text{NH}_3^+$	Evaluation of internalization and inflammatory potential	Mouse	i.cr. 500 ng	Weak transient infl.	Bardi et al. [229]
	Shortenting + oxidation + $-\text{NH}_3^+$				Moderate transient infl	

C ₆₀ fullerene	–	Neuroprotection	Rat	g.i. 3 µg/kg/die	No	Tykhomyrov et al. [192]
–	–	Recovery of neuronal functions	Aβ amyloid AD model rat	i.cr. 5 µg	No	Podolski et al. [193]
–	–	Toxicity analysis	Rat	i.c.v. 0.25 mg/kg	Yes (moderate)	Yamada et al. [194]
				i.p. 0.25 mg/kg	No	
Carboxylic acid (¹⁴ C-labeling)		Toxicity analysis	Rat	i.p. 500 mg/kg	Yes	Yamago et al. [195]
				Biodistribution analysis	i.v. 0.47 mg/rat (est)	No
Tris (malonic acid) (carboxyfullerene)		Neuroprotection	ALS model mouse	i.p. (cont.) 15 mg/kg/day	No	Dugan et al. [196]
Tris (malonic acid) (carboxyfullerene)		Neuroprotection	Fe ²⁺ -induced PD model mouse	i.cr. 3.7 µg	No	Lin et al. [297]
Tris (malonic acid) (carboxyfullerene)		Neuroprotection	MCAO stroke model rat	i.c.v. 0.1 mg	No	Lin et al. [197]
				i.c.v. 0.3 mg	Yes (severe)	
				i.v. 6 mg/kg	No	
Tris (malonic acid) (carboxyfullerene)		Neuroprotection	Mouse	g.i. 10 mg/kg/day	No	Quick et al. [198]
Tris (malonic acid) (carboxyfullerene)		Neuroprotection	Mouse	i.p. 6–40 mg/kg/day, 3 day adm.	No	Tsao et al. [199]
Tris (malonic acid) (carboxyfullerene)		Recovery of neuronal functions	MPTP-induced PD model monkey	i.p. s.c. (cont.) 3 mg/kg/day	Low	Dugan et al. [200]
Hexa (sodium butylsulfonate)		Neuroprotection	MCAO stroke model rat	i.v. 0.1–100 µg/kg	No	Huang et al. [201]
PVP coating		Recovery of neuronal functions	Cycloheximide memory impaired rat	i.cr. 1.7 µg	No	Podolski et al. [202]

(Continued)

TABLE 22.1 (Continued)

Carbon nanomaterial	Chemical modifications	Application	Animal model	Administration route and dose	In vivo toxicity	References
Gd@C ₈₂ fullerene	–(OH) ₂₄ (fullerenol)	Neuroprotection	MCAO stroke model normotensive and hypertensive rats	i.v. 0.5 mg/kg	No	Fluri et al. [203]
				i.v. 1.0–2.0 mg/kg	Yes (severe)	
	–(OH) ₂₄ (fullerenol) + glucosamine	Neuroprotection	MCAO stroke model normotensive and hypertensive rats	5 mg/kg (eq to 0.5 mg/kg fullerenol)	No	
	–(OH) ₂₄ (fullerenol)	Toxicity analysis	Rat	i.c.v. 0.25 mg/kg	Yes (severe)	Yamada et al. [204]
	Amantadine	Recovery of neuronal functions	Haloperidol-induced PD model rat	i.p. 10 mg/kg	No	Nakazono et al. [205]
GO	Gd ³⁺ endohedral –(OH) _n	Tumor imaging	Glioma-bearing rat	i.v. ≤ 9.7 mg/kg	No	Shevtsov et al. [206]
				i.v. ≥ 12.5 mg/kg	Yes	
GO	¹⁸⁸ Re-labeled	Biodistribution analysis	Mouse	i.v. 1–10 mg/kg	No	Zhang X. et al. [207]
	–	Biocompatibility analysis	Mouse	i.v. 100 µg	No	Qu et al. [208]
			Tween 80 coating	i.v. 200 µg	No	
	Dextran coating	Biocompatibility analysis	Mouse	i.v. <125 mg/kg	No	Kanakia et al. [209]
				i.v. ≥ 125 mg/kg	Yes (low)	
	PEG	Imaging	Mouse	i.cr. 40 ng	No	Qian et al. [210]
	PEG + Fe ₃ O ₄ NPs + epirubicin	Targeted drug delivery	Mouse	i.v. + LFUS 0.5 µg	No	Yang et al. [314]

	Gd-DTPA + PAMAM + epirubicyn + miRNA (Let-7)	Imaging drug delivery	Mouse	i.v. dose n.a.	No	Yang et al. [315]
	PEG + transferrin + doxorubicin	Targeted drug delivery	Glioma-bearing rat	i.v. 5.6 mg/kg	No	Liu et al. [211]
	PET + Tat-peptide + perfenidone	Drug delivery	Subarachnoid hemorrhage model mouse	i.c.v. 20 µg.	No	Yang et al. [316]
NDs	Doxorubicin	Drug delivery	Glioma-bearing mouse	i.cr. 30 µg	No	Xi et al. [212]
	—	Toxicity analysis	Mouse	i.cr. 1 µg	No	Huang et al. [213]
SWCNHs	QDs + Gd3N@C80	Imaging	Glioma-bearing mouse	i.cr. dose n.a.	No	Zhang et al. [214]
CNFs	Impregnated with stem cells	Neuroregeneration	MCAO stroke model rat	i.cr. dose n.a.	No	Moon et al. [215]
	Graphitized + impregnated with stem cells			i.cr. dose n.a.	No	
CDs	—	Imaging	Mice	i.v. 100 mg/kg	No	Qian et al. [216]
	—	Imaging	Glioma-bearing mouse	i.v. 100 mg/kg	No	Ruan et al. [217]

The table summarizes the findings of a variety of studies in the related field; it includes the type of the nanomaterial utilized, the chemical modifications attempted, the animal models used in the study, along with the administration route and the in vivo toxicological profile uncovered. ALS, amyotrophic lateral sclerosis; BBB, Blood–brain barrier; CDs, carbon dots; CNFs, carbon nanofibers; DOX, doxorubicin; Gd-DTPA, gadolinium-diethylenetriamine pentaacetic acid; GO, graphene oxide; LFUS, low frequency ultrasound; MCAO, middle cerebral artery occlusion; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine; MWCNTs, multiwalled carbon nanotubes; NDs, nanodiamonds; NPs, nanoparticles; PAMAM, polyamidoamine; PD, Parkinson’s disease; PEG, poly(ethylene glycol); PET, positron emission tomography; PVP, polyvinylpyrrolidone; QDs, quantum dots; SWCNHs, single-wall carbon nanohorns; SWCNTs, single-walled carbon nanotubes.

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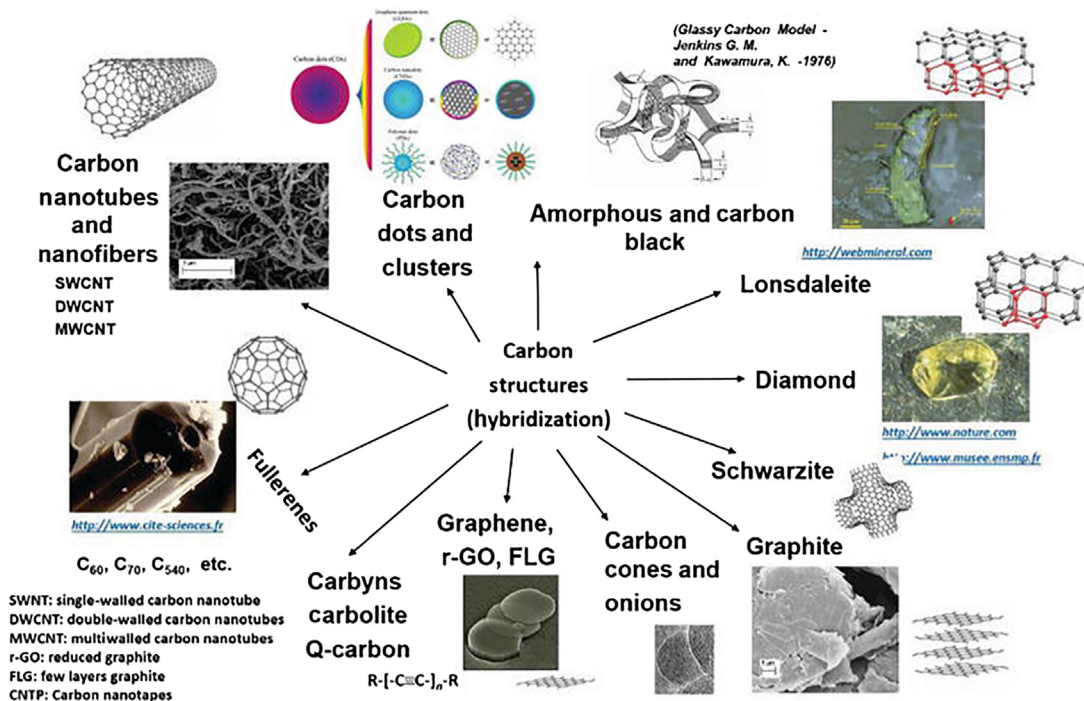


FIGURE 22.3 Illustrative demonstration of various carbon nanostructures. (a) Fullerene (0D); (b) carbon nanotube (CNT) (1D); (c) graphene nanoribbon (1D); (d) graphene sheet (2D); (e) graphite (3D); (f) 3D CNT networks (3D); (g) hybrid of graphene with horizontal CNT (hCNT) (3D); (h) hybrid of graphene with vertical CNT (vCNT) (3D); and (i) graphene triple periodical minimal surfaces (or schwarzites 3D graphene).

from 2 to >30 nm for MWCNTs, while their length can vary from a few hundreds of nm to several hundreds of microns. They have been widely studied *in vivo* and used in research for possible CNS diagnostic and therapeutic applications due to their mechanical, thermal, and electrical properties [219–225]. CNTs primarily offer long-term cues for neurite outgrowth (e.g., for applications like post-SCI or brain injury neuroregeneration), functioning as implants [226]. Both SWCNTs and MWCNTs have a role in neural tissue engineering applications. SWCNTs are mainly preferred as substrates aiming at neural cells modulation and stimulation. This is achieved through variation of conductance, just like it has been attempted using lateral currents for healing neurological injuries [175]. In *in vitro* studies, SWCNTs are also utilized in order to act as substrates for inducing neurite outgrowth [174,227,228]. Conversely, MWCNTs, due to greater stability, have been mostly used as neural guidance conduits and targeted drug delivery carriers [229,230]. An additional application of CNTs regards the implementation of CNT-based electrodes into chronically implantable neural interfaces and recording of electrogenic cells [115,231].

Overall, CNTs have demonstrated good compatibility with the CNS tissue. Several studies have used CNTs for their neuroprotective and neuroregenerative effects against post stroke neurodegenerative changes, as well as their drug and nucleic acids delivery

capabilities [190,232–239]. Despite the promise that CNTs hold for neural tissue engineering applications, there are still concerns for their toxicological profile [240–245]. In general, CNTs-related toxicity has been found to vary depending on the CNTs' administration site, on their agglomeration state (especially nonfunctionalized CNTs tend to aggregate in bundles similarly to asbestos fibers, triggering an inflammatory reaction) [246] and on the presence of metal impurities (Fe, Ni, Co, and Y NPs) in the material.

In terms of the CNTs output, CNTs are either eventually excreted via the urine, or they get accumulated in secondary organs, staying indefinitely in case of a failed attempt of the immune system to eliminate them [247]. Czarny et al. demonstrated that their biopersistence was up to 12 months, without a decrease [248]. Functionalization, dispersion, and length are all parameters affecting CNTs' biodistribution. For example, there is a tendency for covalently functionalized CNTs to be excreted through the urine, contrary to pristine and noncovalently functionalized CNTs that tend to accumulate in the liver and spleen [247]. Dispersion is another important factor for CNTs' toxicity; using small hydrophilic groups like PEG allows good and stable CNT dispersion, improving the pharmacokinetic behavior of CNTs and decreasing toxicity. Nevertheless, the purification or activation by oxidation processes that are prerequisites for most functionalization processes to take place, significantly shorten certain CNTs (especially MWCNTs); therefore, the extent of the influence of the length and functionalization on the CNTs' toxicity is hard to be determined. Another issue contributing to the CNTs' toxicity is the formation of CNT bundles or agglomeration. Bigger agglomerates accumulate quickly, possibly damaging neighboring cells. They are easily detected by the immune system, but the body still has inadequate mechanisms to properly engulf them through macrophages, therefore phagocytosis is compromised and they cannot be cleared [247,249]. In general though, concerns regarding the toxic effects of CNTs secondary to their accumulation inside the body have been mitigated because of studies supporting that CNTs can be enzymatically degraded by peroxidases [250,251] in macrophages [252], eosinophils [253], neutrophils [254], and microglia [255], as well as in the extracellular space [256].

In experiments involving neuronal cells that are traditionally thought to be sensitive to toxins or inflammatory reactions, CNTs' use seldomly show toxicity toward the brain tissue [257–262]. This is probably because the CNTs used for such applications are highly purified and have been functionalized through covalent or noncovalent functionalization with polar moieties to accomplish high dispersibility [263]. However, from the clinical translation standpoint, a big issue that hampers a cost-effective and large-scale production of highly purified CNTs is the costly and complicated procedures and functionalization techniques [264,265] required for the elimination of the catalyst metal NPs.

Overall, CNTs are extremely versatile biomaterials for numerous useful applications in the CNS. Depending on their single-wall or multi-wall nature they can induce different responses [266] and be favored for different applications. With appropriate functionalization techniques CNTs can turn into great candidate biomaterials to induce a good therapeutic response after an ischemic damage [184,267], as well as become excellent vectors for drug delivery applications in the CNS [187,191,259]. Contrary to some studies involving administration to the lungs and gastrointestinal (GI) tract, CNTs show in general high biocompatibility with the brain tissues [268]. CNTs have even been able to mitigate the toxicity of some drugs. In summary, CNTs are considered to be cutting-edge

nanomaterials for the therapy of CNS diseases and they could also be combined with the use of hydrogel systems in order to allow for a more controlled exposure of the tissues in an attempt to eliminate toxic reactions with proper optimizations. Further studies in order to test the toxicological profile of CNTs using different functionalization techniques and tunable delivery systems are still highly important in order for CNTs to be considered safe candidates for clinical use in the future.

22.5.2.2 Fullerenes

Fullerenes [269] are defined molecular entities with a precise atomic composition and hollow spherical shape, with C₆₀ fullerene (also called buckminsterfullerene) being the most widely studied in this biomaterials' category. C₆₀ fullerene is in fact the first and the smallest stable fullerene isolated and the relative ease of synthesis is attractive to the scientific community. It is obtained in relatively good yields from graphite using the arc-discharge technique. Subsequently, it is purified from by-products by solvent extraction, followed by chromatography. In terms of structure it represents a truncated icosahedron structure with a diameter of 0.7 nm due to the 60 sp²-hybridized carbon atoms' arrangements in space. Depending on the application required, certain fullerenes' derivatives have been developed with hydrophilic (carboxyfullerenes) or lipophilic (phenyl-C61-butyric acid methyl ester) behavior in order to increase the solubility in water and organic solvents.

In the last 30 years, just like CNTs, fullerenes had an important place as a cutting-edge nanomaterial for a wide range of biomedical applications. Several studies have explored their use as oxidative damage protecting agents, photosensitizers for photodynamic therapy of cancer, antiretroviral agents, and as drugs and gene delivery vectors [192,270–273] among others. Fullerenes were even studied in an *in vivo* level to test potential applications for the treatment of brain disorders. Nevertheless, the interest of the scientific community for biomedical applications has subsided due to toxicity-related concerns. Specifically, pristine C₆₀ displays just moderate toxicity [271,274–276], but its covalent and noncovalent derivatives can be very toxic [277,278]. In addition, the presence of surfactants or organic cosolvents increases the toxicity of pristine C₆₀ [276], while genotoxicity has also been reported [279].

Fullerenes have also shown good potential for CNS applications, given their BBB crossing capabilities. In fact, they were the first carbon nanomaterials that were found to distribute in the brain after systemic administration in *in vivo* studies [189] without toxic effects, contrary to outcomes from different routes of administration (e.g., intraperitoneal); this difference in toxicity is possibly related to the biodistribution. The importance of the route of administration has been demonstrated in many studies. For example, despite the fact that carboxyfullerene displays a nonnegligible toxic profile for the CNS tissue, the systemic administration drastically reduces toxicity. Most importantly for potential clinical implementation, continuous systemic administration (3 mg/kg/day) using either intraperitoneal or subcutaneous osmotic pumps, has been tested for carboxyfullerene in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treated nonhuman primate models for the treatment of PD [200]. The results indicated that continuous administration of carboxyfullerene was in fact capable of inducing recovery of dopaminergic neurotransmission even after initiation of neuronal death processes. However, to reach a neuroprotective effect, chronic administration is required that raises toxicity-related questions given the scarcity of long-term toxicological data.

Overall, they have been favored as neuroprotective agents [196,198,201,280–282], whereas their use in the CNS as drug delivery vectors or imaging agents has been just marginally explored [206,206]. They have demonstrated great potential with good results for CNS biomedical applications, but fullerenes are still considered history for the field due to their proven accumulation in several organs, their long biopersistence and, in general, their unpredictable toxicity [276]. In particular, water-soluble derivatives which are the most appealing for integration into drug products demonstrate more evident toxicity compared to nonfunctionalized fullerenes, severely limiting fullerenes' practical use for the development of therapies. Even the fact that they can be synthesized in large quantities at relatively low costs does not compensate for their toxicological profile and limitations so far.

22.5.2.3 Graphene oxide and derived nanomaterials

Graphene is an allotrope of carbon, which consists of a single layer of carbon atoms arranged in a 2D hexagonal lattice. Graphene oxide (GO), the most common derivative of graphene and the most preferred form of graphene used for biomedical applications, is being produced from the exfoliation of graphite by oxidation procedures. Graphene's heat and electrical conductivity, near transparency, bactericidal and antiviral activity, as well as its high biocompatibility with low cell toxicity are properties that make graphene a very attractive choice as a biomaterial for CNS biomedical applications (e.g., toxicants and tumor marker sensing, in vitro and in vivo imaging applications, drugs and nucleic acid delivery, tumor photothermal ablation, as well as stem cell differentiation substrates) [260,283–293]. On top of that, GO can exhibit inherent and tunable optical absorption and emission properties, with emission wavelengths varying from near-infrared (NIR) to blue light, depending on the size, composition, and degree of oxidation [294–297], something very useful for biomedical applications. Three-dimensional cultures offer an advantage compared to the 2D ones due to some differences noted in the interaction of graphene with cells in those settings. Specifically, some matrix cellular toxicity has been reported [298,299], with three-dimensional cultures demonstrating better neural cell growth and proliferation. Another advantage offered by three-dimensional graphene substrates is their capability of encapsulating various NPs (e.g., gold), increasing neuronal differentiation and guiding axonal alignment [300].

The use of graphene for neural tissue engineering applications has involved a wide range of graphene's forms. Foams and nanogrids made of graphene have shown promise for neural tissue engineering. Rolled graphene foams is one example of graphene-based three-dimensional scaffolds with great electroconductive properties that have stimulated and accelerated differentiation and proliferation of human NSCs [301,302]. In addition, graphene nanogrids have been shown to increase the neural cell to glial cell ratio, upon application of biocompatible stimulation techniques (e.g., electrical, pulsed laser, flash photo, NIR laser stimulation) [303–306]. Finally, neural-device interface enhancement is another aspect made possible thanks to graphene-based neural probes [307].

Graphene has been called the miracle material due to its unique properties; therefore, it does not come as a surprise when many studies have reported graphene and its derivatives as suitable candidates for both diagnostic and therapeutic CNS applications. Their intrinsic fluorescence, permeability of brain tissue despite the BBB (even though low per se for not functionalized graphene), their high loading capacity of biomolecules, drugs or

imaging agents, and, yet, their lack of CNS toxicity, are just some characteristics that facilitate neural tissue engineering applications. Several reports support the use of graphene as a permissive substrate for neuronal cells growth [302,308–313]. In an attempt to guide the elongation of neuronal processes in a controlled way, the electroconductivity of graphene has also been exploited [308]. Unfortunately, functionalization with high efficiency targeting molecules or the employment of novel physical BBB opening techniques is absolutely necessary in order to overcome the problem of low brain tissue permeability. This nanomaterial has still been in an infantile stage of development for CNS applications; therefore, adequate optimizations have not been established in terms of size, functionalization, dose, etc. to accomplish the best possible outcomes. For the same reason, the toxicological profile of graphene has not been deeply investigated along with the parameters affecting toxicity (e.g., size, functionalization, etc.). However, the attention given to graphene and its promise, nowadays, is definitely meant to bring more answers to those topics and, thereafter, rapid improvements in the field.

Overall, GO and derivatives can be successfully used for CNS tissue engineering applications, as well as for imaging applications, by exploiting their intrinsic NIR fluorescence or the functionalization with magnetic resonance imaging (MRI) contrast agents. Targeted delivery of therapeutic drugs and genetic materials have been made possible inside cerebral tumors [211,314–316]. On the other hand, in order to accomplish BBB permeability, appropriate functionalization or physical BBB opening methodologies are mandated [315]. Graphene's toxicity profile is, as for many other carbon nanomaterials, highly dependent on the functionalization, size, and the aggregation behavior [317–319] with GO being considered the less toxic derivative compared to pristine graphene, reduced GO, or hydrogenated graphene. Additional functionalization (with PEGs, aminogroups, etc.) reduces the toxicity even further. The accumulation of graphene in lungs and the longer persistence in the body compared to other carbon nanomaterials raise concerns [207,209,298,320–324], but graphene's low-toxicity profile, especially in the presence of extensive appropriate functionalization, allows for some promise in terms of potential future implementation in certain therapeutic biomedical applications.

22.5.2.4 Nanodiamonds

Nanodiamonds (NDs) are carbon nanomaterials formed by sp^3 carbon atoms arranged in a diamond-like cubic lattice. Their diameter can range anywhere from 4–5 to 100 nm. NDs synthesis is usually performed at high pressure-high temperature with detonation of trinitrotoluene and nitroamines (research department explosive) being the most used production method developed [325,326]. NDs' surface is highly reactive and can be easily functionalized or passivated [327–330].

It has not been long since NDs have been proposed as suitable nanomaterials for biomedical applications (e.g., bioimaging [328,331–333], drug delivery, and nucleic acid delivery [329,333–335]), exploiting the functionalization with targeting molecules for improved selectivity [336,337]. NDs' use is undeniably in an infantile stage with limited studies compared to those available for other carbon nanomaterials. Studies on NDs' toxicity are also very limited at that moment [213,338]. Nevertheless, NDs have already shown promising drug delivery capabilities inside the CNS [212]. Neuronal cells cultured on a surface of NDs reveal cell growth and electrophysiological properties comparable to neurons grown on classical

supports [339–342]. When administered *in vivo*, NDs accumulate in the liver, in the spleen, and in lymph nodes [343,344]. So far, NDs have not displayed any alarming toxicity. Although the research on possible diagnostic and therapeutic applications of NDs in the brain is in the early stages, the current results suggest that these nanomaterials may have an important role to play in the future CNS tissue engineering therapeutics.

22.5.2.5 Carbon nanohorns and carbon nanofibers

Single-wall carbon nanohorns (SWCNHs) are carbon nanomaterials that are structurally similar to CNTs, but they remain relatively unexplored, especially in biological studies. They differ from CNTs because of their continuous graphitic surface that is arranged in a conical shape with a closed tip. They are usually 40–50 nm long and 2–3 nm wide, and they commonly assemble into 80–100 nm spherical aggregates [345,346]. SWCNHs have been used for certain biomedical applications with good *in vitro* and *in vivo* results on areas like biomolecule sensing [347], MRI imaging (as support) [348], photodynamic and photothermal therapy of cancer [349–351] as well as drug and gene delivery [348–353].

On the other hand, CNFs are tubular carbon nanostructures, with diameters in the range of 3–100 nm and lengths that can also exceed 1 cm [352]. Long fibers that are often hollow are formed through the assembly of curved graphitic layers. They are essentially made of assembled curved graphitic layers arranged in different ways to form long fibers, often hollow. Biomolecules sensing [353–357], gene delivery [358], and regenerative medicine [359,360] are only a few areas where CNFs have been used. Nevertheless, just like it happened with the CNTs, toxicity-related concerns have been raised, including the non-biodegradability and accumulation in organs like the lungs [361,362].

CNFs have demonstrated good biocompatibility with neuronal cells, favoring neuronal versus glial/astrocytic proliferation, taking a place as coating nanomaterials for neural prosthetic devices [359,360]. They have also been found to provide mechanical, chemical, and electrical cues in order to support and guide neuronal cellular growth, but they have also been involved in microelectrode arrays for *in vivo* signal detection and manipulation [363–367].

Despite the structural similarities between SWCNHs and CNTs, the SWCNHs' synthesis is metal-free, eliminating potential toxic effects secondary to metal contaminants. Toxicology-related studies have been conflicting and relatively limited so far with some indicating biocompatibility regardless of the accumulation to organs like lung, spleen, and liver [368–370], and some others supporting SWCNHs' toxicity towards macrophages even at low doses [371]. This does not allow for conclusions to be drawn from those studies until further studies investigate the toxicological profile of SWCNHs.

The use of SWCNHs and CNFs in imaging- and delivery-related biomedical applications has been severely limited by the inability of those materials to cross BBB due to their relatively big size [370,372]. This limitation can be overcome by using *in situ* administration of the materials, but this increases the risks by requiring more invasive procedures for the administration (e.g., intracranial administration). The concerns for toxicity and accumulation in several organs have also been problematic. Therefore, the utility of SWCNHs or CSFs in CNS diagnosis and therapy has been limited to the extent

of development of electrode or composite nanostructures for stimulating and guiding neuronal growth.

22.5.2.6 Carbon dots

Carbon dots (CDs) are a recently discovered class of quasispherical carbon-based nanomaterials [373]. They combine the presence of an amorphous or nanocrystalline (Csp^3) core and a graphitic or turbostratic (Csp^2) shell. The industrial scalability of their production has been problematic despite the many synthesis approaches used (e.g., top-down, bottom-up, etc.) [374–377]. The CDs' high intrinsic fluorescence, spanning from the visible to the NIR [376,378], can be very useful for bioimaging applications [217,374,379–381], even though artifacts can be promoted, especially when other luminescent probes are being used in parallel [382–385]. Drug delivery applications have also utilized CDs by noncovalently loading biomolecules, drugs, or nucleic acids on their surface [386,387]. The high stability of the CDs' suspensions in water and biological fluids, potentially due to the presence of highly dense charged groups on their surface has been invaluable for biomedical applications, offering a very good biocompatibility profile [216,217,388–395].

CDs are being eliminated fast from the brain tissue, but they also do demonstrate spontaneous BBB crossing capabilities, which is very important for CNS neural tissue engineering applications [216]. Being in the very early stages of their development, certain optimizations using functionalization techniques might help scientists address any limitations. For example, opportune chemical modifications with molecules could increase their plasma circulation time and/or targeting moieties could improve their retention in the brain, allowing CNS applications like tumor therapy [217]. The current toxicological data would not be adequate to draw conclusions on their safety and clinical translatability. A significant limitation is currently the fact that large-scale industrial production has not been technologically possible, regardless of the relatively easy and cheap production in the laboratory scale, even though the synthesis is relatively easy and cheap on a laboratory scale. A deep toxicological evaluation of their effects in the CNS, but also in the whole body would be certainly needed for future advancements in the field [384,390,395–398]. Given the fact that the effect of CDs on cellular biochemistry has not been completely unraveled, caution is advised for investigations suggesting potential future clinical applications.

22.6 Conclusion and future directions

Targeting the CNS in order to structurally bridge any lesions and promote neuroregeneration within a hostile environment is a challenging task. The only way to maximize the chances of success is to get familiarized with the properties of the different kinds of biomaterials that are appropriate for CNS neural engineering applications in order to pick the right candidate for the desired task. Combinatorial approaches with the emergence of novel composite hydrogel systems can allow an even better level of control and tunability of the matrices to maximize the chances of successful CNS nerve repair. This is significant, given the fact that there is no “perfect” biomaterial that would fully match the profile and characteristics needed for a neural tissue engineering intended

application. Overall, the field of neural tissue engineering is a rapidly advancing field with tremendous potentials for the future of patients with CNS disorders. It is anticipated that, with that pace, the future management of CNS disorders could already be different after the next decade with the introduction of new regenerative treatment strategies. Of course, such strategies might hold promise for a bright future, but, currently, they are still in an infantile stage in terms of clinical translation. Thus, caution should be exercised for biomaterials that might show promise in vitro or in vivo in rodent models and smaller animals, because there is no direct clinical translation path without the use of nonhuman primate models and without comprehensive studies focusing on neurotoxicity and biocompatibility. In order for those promising biomaterial candidates to safely reach the clinic as novel regenerative therapies for CNS conditions, such studies are mandated, so that the possibility of obtaining a desirable CNS response rather than dangerous adverse reactions gets maximized and, therefore, the risks do not outweigh the benefits for future human subjects in early clinical trials.

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Conflicts of interest

The authors declare no conflicts of interest relevant to this work.

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Peripheral nervous system responses to biomaterials

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23.1 Introduction

Peripheral nerve injuries (PNI) typically involve the upper limb and are often associated with trauma [1]; other causes include tumor resection and iatrogenic injury during surgery for other diseases. Traumatic PNI disproportionately affects young and working members of the population and can lead to functionally devastating consequences with loss of sensation and function, pain, and cold intolerance [2] leading to significant socioeconomic problems to both patients and society.

Treatment of a completely transected nerve aims to maximize its regeneration potential through restoration of continuity of the severed ends as quickly as possible [3,4]. This involves mobilization of the cut ends of the nerve, debridement of any interposed scar tissue or debris back to healthy fascicles. Then using meticulous microsurgical repair techniques the nerve stumps are loosely approximated using fine epineurial or fascicular (perineurial) sutures [5,6] (Fig. 23.1).

Some injury patterns create a gap [14] between the severed nerve ends that prevents direct repair. In this instance an alternative to direct repair is required as epineurial approximation under tension leads to poor outcomes [15]. When a nerve gap exists the current “gold standard” treatment is a reversed, autologous nerve graft [16]. This provides a scaffold with readily available neurotrophic factors and Schwann cells (SCs) to help

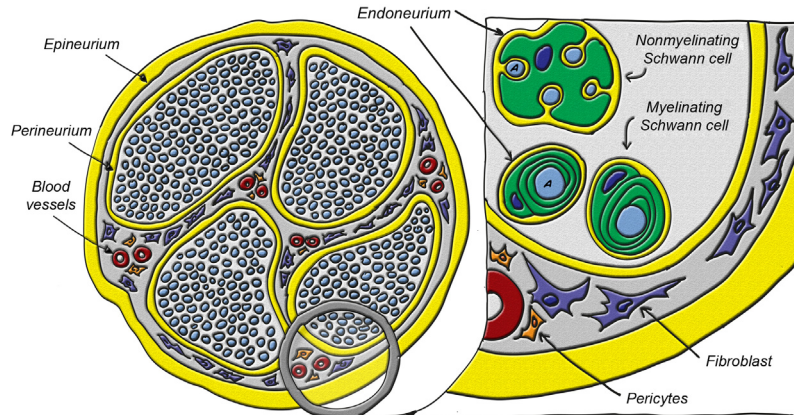


FIGURE 23.1 Cross-sectional anatomy of a cut nerve end.

The functional unit of a peripheral nerve is the neuron which has a nerve cell body in communication with the central nervous system (the spinal cord) and distributes signals to its target organ via a myelinated or unmyelinated axon. Myelin is a predominantly fatty layer with relative electrical resistance and low capacitance that insulates the axon and encourages longitudinal conduction of the action potential [7]. Support cells of the peripheral nervous system, known as SCs ensheath the axon and provide a crucial role in the maintenance and regeneration of neurons [8]. These axon-SC complexes are enveloped in a layer of ECM called the endoneurium which is rich in laminin [9]. These endoneurial tubes are grouped together into nerve fascicles which are surrounded by perineurium. Perineurium acts as the main barrier between the endoneurium and extra fascicular blood supply, with vessels perforating obliquely from the epineurial network to supply the endoneurial structures [10–13]. Perineurium provides protection to the underlying endoneurial tubes through its ability to modulate external stretching forces due to its composition of fibrillar and microfibrillar collagens and fibronectin [9]. It is surrounded by a basement membrane containing Type IV collagen and laminin providing structural support [11]. Surrounding the groups of perineurial-lined fascicles is the outermost layer of the nerve, the epineurium. This is formed mostly of collagenous ECM and provides tensile strength to the nerve.

guide axonal regeneration while also preventing the formation of a neuroma and the ingress of scar tissue, which could block axonal growth [17].

Over the past 80 years there has been a great effort made to develop an alternative to the nerve autograft in order to avoid the donor site problems and limited supply of nerve autografts, while attempting to retain the same degree of nerve regeneration. This goal has led to the development of a subset of biomaterial research, often combining the expertise of scientists and nerve surgeons working in collaboration to produce a greater understanding of the requirements of a nerve guidance conduit (NGC). The overarching aim is to discover an immunologically inert biosynthetic conduit with regenerative properties comparable to autologous nerve grafts.

Ideally the NGC should be tube-like to allow attachment of either end of the injured nerve and allow axonal growth along the tube while providing protection from external scar tissue which could block the developing growth cones. The walls of the tube need to be porous and permeable to allow the diffusion of oxygen, metabolic substrates, and growth factors required by the regenerating nerve. It must be flexible to allow movement especially if it is to be placed across a joint, while also maintaining enough rigidity to prevent collapse and blockage of the

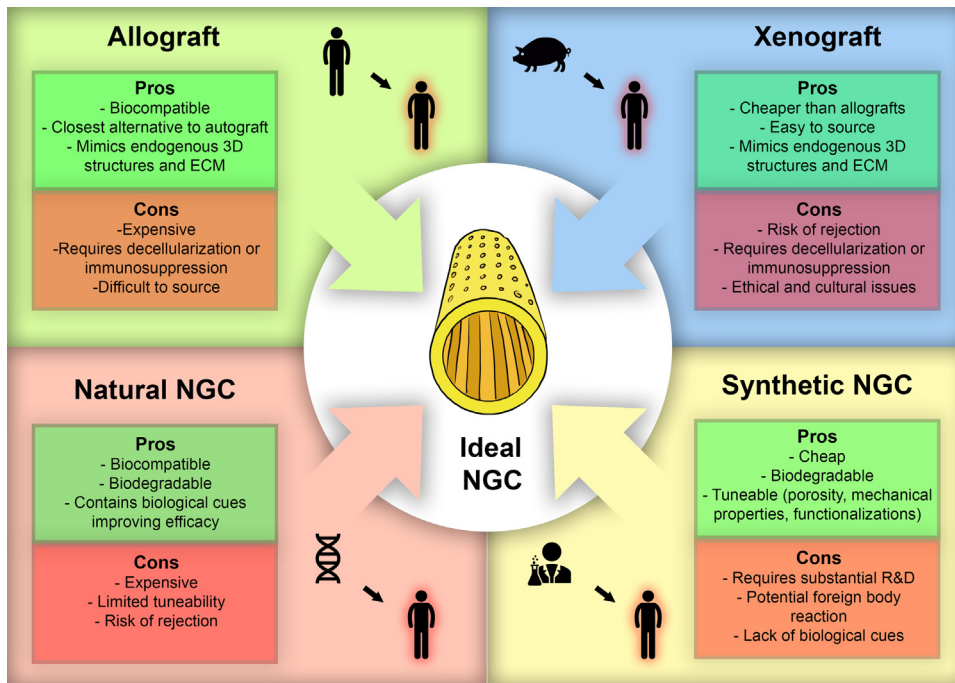


FIGURE 23.2 Alternative biomaterial approaches to the ideal nerve conduit.

tube. It should be biocompatible and biodegradable in order to reduce the unwanted immunological response from the body when implanted, in addition the internal lumen material should not inhibit axonal growth and instead, should seek to enhance it [18,19].

Current research has led to the development of a plethora of alternative tubulization biomaterials which can be divided into: nonsynthetic NGCs and synthetic NGCs with subdivisions depending upon the exact material used (Fig. 23.2). These will be discussed in much greater detail below.

23.1.1 Non synthetic nerve guidance conduits

23.1.1.1 Autografts

In the later part of the 19th century nerve surgeons experimented with NGCs made from decalcified bone, however the results were very poor and they soon sought alternatives [20]. Tubed anatomical structures were sought and the use of autograft blood vessels and skeletal muscle showed promising results in animal and human experiments.

23.1.1.2 Blood vessels

As an alternative to nerve autografts, blood vessels provide the most suitable, abundantly available tube-like structures in the body. Arteries contain large amounts of laminin within the endothelial layer and media layer [21] which is known to be an important factor

for neurite outgrowth [22,23]. In addition to the laminin-rich endothelium, the outer adventitial layer of vessels is composed primarily of collagen which provides structural support and prevents scar tissue ingrowth while being porous enough to allow for the diffusion of nutrients [24,25]. Vessels, therefore, appear to provide a good alternative to a nerve autograft.

Weiss described the use of an arterial segment as a NGC in an experiment [26] in rats. This initial case-report was undertaken prior to the advent of the operating microscope and so a “tight fitting” arterial segment was used without suture anchor to the proximal or distal nerve stumps. More recently [21,27] arterial autografts have demonstrated to have similar regrowth potential when compared with nerve autografts across small segment defects (<3 cm) in rats. However, their clinical applicability remains limited owing to the difficulty in safely harvesting an appropriately sized (both diameter and longitudinally) artery for corresponding nerve defects.

Subsequently, interest amongst researchers and clinicians has moved toward the use of veins instead which can be more safely harvested, and veins contain a similarly high level of laminin on their endothelial surface to arteries. Experiments in rats comparing vein autografts and nerve autografts have demonstrated similar regeneration potential [28,29] and they have been validated for clinical application in nerve gaps up to 3 cm [30–32].

The main disadvantage of vein grafts is the presence of valves in the lumen, which may obstruct axonal outgrowth with the potential to form a neuroma. This problem can be avoided by selecting a valve-less section of donor vein or the vein conduit can be inverted to an inside-out orientation as described by Wang et al. [24]. Another disadvantage to the use of autologous vein grafts is the potential for the tube to collapse thereby impeding axonal regrowth. This is more likely to occur in larger nerve gaps >3 cm and can potentially be avoided by filling the vein with material that acts to splint open the tube and also enhance axonal regeneration. Several different filler materials have been trialed including nerve, muscle, and platelet-rich plasma [33–35] which can supply important neurotrophic elements of the extracellular matrix (ECM) and neurotrophic factors which promote SC migration, cell proliferation, and axonal growth-cone guidance.

23.1.1.3 Muscle

Muscle has been used as both an insert in vein-tubes or as a NGC on its own. When inserted into a vein-tube it provides structural support to prevent collapse and neurotrophic factors and ECM proteins (mainly laminin) which encourage SC migration and guidance of growth cones [35]. When used as a NGC on its own, the longitudinal orientation of the ECM and basal lamina enhances regenerating axons [36]. However the main disadvantage of muscle NGCs is a lack of a structurally robust enveloping layer made of collagen (that is present in vessels). This increases the risk of neuroma formation as the regenerating axons may easily grow through the muscle fibers into surrounding tissue unimpeded. In addition, as with any autograft, a donor site to harvest the muscle is still required.

Autograft alternatives to nerves such as veins or veins filled with muscle have demonstrated a suitable alternative over small gap sizes (<3 cm) and certainly have a role to play in small sensory nerve gaps in the hand as they are easily harvested and secured in

place. However they still leave a donor site which could be significant if larger amounts were required.

23.2 Allografts

Peripheral nerve allografts require either preprocessing in order to remove their immunogenic material or immunosuppression of the host in order to maintain graft survival. Commercially available processed nerve allografts (PNAs) from deceased human donors have had immunogenic cellular and noncellular components removed via a process of chemical decellularization and gamma irradiation [37] leaving a sterile and decellularized ECM three-dimensional (3D) scaffold with a basal lamina tubular structure. They have been approved by both the United States Department of Food and Drug Administration (FDA) and National Institute of Clinical Excellence for use in small (<3 cm) sensory nerve gap repairs [38]. In a prospective, nonrandomized, comparative study from China [39] ($n = 153$) their PNA had significantly better two-point discrimination ($P = .003$) than the direct repair group at 6 months, but no difference in Semmes-Weinstein monofilament testing. PNAs are revascularized after insertion owing to their porous basal lamina structure which provides continued nutritional support to the infiltrating SCs.

Research in animal models has demonstrated a potential target for immunosuppressive therapies in order to allow nonprocessed allograft survival [40], however its transition to human trials has never materialized owing to the success and improved cost efficiency of preprocessing techniques which produce nerve allografts that do not require host immunosuppression.

Commercially produced PNAs are available in the clinic [41] and with the lack of donor site they are an important clinical option, however they are currently very expensive to produce and are therefore limited to use in adults, in specialist centers in the United Kingdom. Further clinical evaluation for their use is currently ongoing with a large multicenter retrospective study analyzing clinical outcomes of PNAs compared with nerve autograft and conduit repairs [42].

23.3 Xenografts

Xenografts have the potential for unlimited supply but have the obvious drawback of immune rejection. In animal studies highly variable outcomes have been reported [43–45] and attempts at human trials around the World War II did not show favorable outcomes [45]. The effects of immune rejection can lead to the deposition of scar tissue which blocks axonal regeneration limiting the gap size for which xenografts can be successfully used on defects less than 10 mm [46]. Attempts to pretreat the grafts in order to reduce the antigen burden have been proposed but these have not led to successful breakthroughs in the use of xenografts [44], partly because they significantly reduce the number of available SCs within the graft. Instead researchers in peripheral nerve xenografting have turned their attention toward developing targeted methods of immunosuppression. Although hyperacute rejection does appear to occur in the xenotransplants, graft rejection is thought to

be due to a cell-mediated response. Some of the molecular components of this rejection have been identified such as interferon-gamma-producing Th1 cells and IL17-producing Th17 cells [47]. It is therefore possible that neutralizing antibodies targeted toward these molecules may reduce nerve xenograft rejection in the future. Despite the hurdles to the clinical use of peripheral nerve, xenografts they have the potential to be a useful resource to nerve surgeons. Whether they develop into a clinically useful tool, however, is yet to be seen.

23.4 Natural degradable nerve guidance conduits

23.4.1 Collagen

Collagen constitutes nearly 50% of extracellular peripheral nerve proteins [48] with a similar ratio of Type I: III (80:20) as the skin [49]. As a synthetic, biodegradable NGC it has some useful attributes given that it is porous, biocompatible, and absorbable [50] but its results in in-vivo experiments of nerve regeneration appear to be mixed. In animal nerve regeneration models it has demonstrated similar outcomes to autograft repair in a 5 mm median nerve gap in primates [51], however in an 18 mm gap in a rat PNI model ($n = 3$) an empty collagen NGC failed to support any axonal regeneration [52]. In another similar rat injury model with a gap size of (10 mm), an empty collagen tube group ($n = 5$) demonstrated only two-thirds of the total axonal counts of the autograft group ($n = 4$) at 30 and 180 days [53]. In another 14-mm gap, rat-sciatic PNI model the collagen NGC performed inferiorly compared to isograft and allograft groups at 6 weeks [54].

Early clinical experience in humans using a Type I bovine collagen NGC demonstrated acceptable outcomes in repair of small (10–20 mm) sensory nerve gaps in the hand [55–57]. In larger diameter forearm motor nerve repairs in humans Dienstknecht et al. [58] and Klein et al. [59] demonstrated satisfactory sensory and patient-reported outcome measures with the use of bovine collagen conduits. Unfortunately, neither prospective trial compared the use of the bovine collagen conduit with a nerve autograft repair and therefore it remained unclear as to whether collagen conduits can perform as well as the current gold standard in larger diameter nerves and in larger gap sizes. Despite this there are a number of Type I bovine collagen-derived NGCs and wraps that have gained regulatory approval for use in PNI including: NeuraGen [Integra, FDA 510(k) approval 2001; CE mark 2003], NeuroMatrix, and NeuroFlex [Stryker, FDA 510(k) approval 2014] [60]. NeuraGen, the longest standing of these, has demonstrated clinical safety and efficacy in small sensory nerve repair [50,56,61] but demonstrated poorer outcomes in mixed motor/sensory nerves [62].

23.4.2 Gelatin

Gelatin, a widely used substance in the food, pharmaceutical, and cosmetic industry, is an irreversibly hydrolyzed form of collagen whereby the collagen protein fibers have been reduced into smaller peptides. Like collagen it is derived from animal body parts but is much cheaper to acquire in concentrated solutions. In order to produce a stable NGC the

gelatin must be cross-linked to maintain its integrity; commonly utilized agents include glutaraldehyde [63] and genipin [64]. This reduces the biodegradation rate and improves surgical handling and fixation [63]. In vivo animal experiments (rats) in a short nerve gap model (10 mm) demonstrated minimal inflammatory response from a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide cross-linked gelatin conduit with complete degradation at 12 weeks and histological evidence of axonal regeneration [65]. The mechanical properties of hydrated gelatin in vivo make purely gelatin-made NGCs a challenge, instead researchers have utilized gelatin to create 3D constructs or scaffolds that can be combined with synthetic conduits in order to seed growth factors and stem cells thereby creating a 3D tubular NGC, with promising results [66,67].

23.4.3 Fibrin

Fibrin, like collagen, is another ECM protein with wide applications in both surgery and bioengineering [67,68]. Fibrin glue can be used as an efficient alternative to suturing of severed nerve ends in direct repair and has shown equivalent regeneration rates in animal models to an epineurial suturing-based approximation technique [69], but no controlled clinical trial has been performed. Fibrin glue can also be used to create NGCs, commonly using a xenogenic mixture of human clotting proteins and bovine antifibrinolytic agent, aprotinin. This can be immunogenic and thus increase the risk of rejection and scar formation, however safety studies in rats and nonhuman primates have demonstrated that the application of fibrin glue to the brain parenchyma, spinal cord, spinal roots, and trigeminal nerve does not appear to induce an inflammatory response or additional damage to nervous tissue [70,71]. In vivo testing of a fibrin NGC has demonstrated complete absorption of a 14-mm fibrin conduit over 15 weeks in rats with similar regenerative results compared to synthetic conduits over this distance [72]. In another rat-sciatic nerve injury model of 10 and 20 mm gaps and compared with autograft, fibrin conduits demonstrated equivalent outcomes to autograft over 10 mm gaps but were much inferior to the autograft group over 20 mm [73]. Its utilization as a NGC appears limited to short gaps (around 10–15 mm), however it is more recently being used to produce fibrin hydrogels, similar to gelatin, to allow for the construct of 3D scaffolds to allow stem cell [74] and growth factor [75] seeding into synthetic conduits.

23.4.4 Keratin

Keratin, derived from human hair and fingernails, is a protein that can self-aggregate into nanofilaments when placed in solution [76], with further self-assembly into a fibrous microarchitecture when in a gel-like state. Similarly to other proteins of the ECM such as collagen, keratin contains multiple peptide binding motifs supporting the attachment of a wide variety of cell types including SCs [77]. Keratin-based hydrogels inserted into synthetic NGCs have demonstrated improved peripheral nerve regeneration in vivo across small nerve gaps compared to empty conduits alone in rat models (<10 mm sciatic nerve gap [61]; 15 mm sciatic nerve gap [78]) and also in nonhuman primates (10 mm median nerve gap [79]).

23.4.5 Silk

Natural silk derived from the silkworm *Bombyx mori* has long been used in the textile industry and also as suture material [80] but did not gain greater utilization in the field of tissue-engineering until early this century [81]. Silkworm silk contains a core structural protein: silk fibroin coated in a glue-like protein: sericin. Sericin has been identified as the cause of adverse immunological responses often attributed to the use of natural silk suture material, and therefore purified silk fibroin that is biocompatible and has useful mechanical properties has been utilized to produce NGCs [81]. Silk fibroin has demonstrated good compatibility with rat peripheral nervous tissue while also proving beneficial for SC attachment and migration [82]. Several in vivo rat experiments have been conducted over the past decade analyzing the regenerative capacity of silk fibroin conduits as empty tubes, seeded with neurotrophic factors or stem cells with promising results [81,83–90]. However, the regenerative capacity of silk conduits alone still appears limited to short (<15 mm) nerve gaps.

23.4.6 Chitosan

Chitosan is a natural polysaccharide derivative of chitin which is structurally similar to natural glycosaminoglycans [91]. It demonstrates good biocompatibility, low toxicity, and is biodegradable [92]. It is a suitable material substrate for SC attachment and growth [93] in addition to neuronal cell survival and growth [94]. One of the main issues preventing in vivo use of chitosan NGCs is their poor mechanical strength due to premature degradation and loss of structural integrity which has been overcome to a degree by refining the acylation chemistry required in their production [95]. This has produced chitosan NGCs that have demonstrated equivalent neuroregeneration to nerve autografts in short (10 mm) rat nerve gap models [96] and in larger (30 mm) dog sciatic nerve gaps when functionalized [97]. Further work is needed to demonstrate their effectiveness in humans.

23.5 Synthetic nerve guidance conduits

Silicone tubes were used in the first generation of artificial NGCs. They are inert, flexible, and readily available; however, as they are not biodegradable or permeable to large molecules they were found to have limited use in peripheral nerve regeneration. It soon became apparent that they led to chronic nerve compression and may damage the regenerating nerves [98]. In addition, as they are nondegradable they present a risk of chronic foreign-body reaction with excessive scar tissue formation [99] and require further surgery to remove the device after nerve regeneration is complete.

This has led to a decline in their use and instead researchers have looked to synthetic degradable NGCs instead. The FDA, who have responsibility for regulatory approval of medical devices for use in the United States have stated that the material used in design criteria for synthetic NGCs must be biodegradable [60].

23.6 Synthetic degradable nerve guidance conduits

Three types of synthetic, degradable peripheral nerve conduit materials have currently been approved for clinical use by the US FDA or European Commission. These are: collagen (Type I), polyglycolic acid (PGA), and poly(lactide caprolactone) which is a copolymer of poly (lactic acid) (PLA) and poly (*ε*-caprolactone) (PCL) [100,101]. Shin et al. [100] compared motor outcomes between a nerve autograft and PCL, PGA and collagen NGCs in a 10 mm sciatic nerve defect in rats. At 12 weeks there appeared to be equivocal outcomes between the autograft and the PCL conduit, with the PGA conduit performing least well having completely collapsed.

23.7 Polymers

Synthetic, biodegradable polymers are relatively inexpensive compared to biological compounds and can be engineered with modified physical and mechanical characteristics such as strength, permeability, and degradation rate as well as cell attachment and proliferation by using physical or chemical modifications [101]. This can allow for the attachment of adhesive proteins, cells, and growth factors, for example, a group from Pittsburgh, USA embedded glial cell line-derived neurotrophic factor-eluting microspheres [made of a poly(lactic-*co*-glycolic) acid/poly-L-lactic acid blend] into the wall of a PCL conduit and demonstrated improved outcomes compared to a conduit-only group over 6 weeks in a 15 mm rat-sciatic nerve defect [102,103].

It has been important to determine which synthetic polymers provide the best tubulization structure and biocompatibility in order to progress their development from animal models to man. Ideally a synthetic conduit needs the mechanical or physical properties that are similar to peripheral nervous tissue in terms of tensile strength, degradation profile, and size, in addition it must also avoid swelling and not elicit an inflammatory response during degradation [101].

There is currently a wide variety of artificial NGCs being explored in animal models more often in small gap defects up to 20 mm. In these smaller gap nerve injury models it is easier to demonstrate regeneration across the gap given the short distances the proximal nerve stump has to travel, and the reduced risk of complications associated with a short NGC. Very few of these animal studies demonstrate reinnervation of the target organ especially in larger gap injuries thereby providing minimal evidence of their effectiveness for use in human trials. Neurotube (PGA) [Synovis, CE mark 1995; FDA 510(k) 1999] and Neurolac (PLA and PCL) are the only two in mainstream clinical use.

23.7.1 Poly (*ε*-caprolactone) (PCL)

PCL, although developed in the 1930s, did not gain popularity until the 1970s and 1980s when it became evident that its rheological and viscoelastic properties allowed for easier manufacture and manipulation into a range of constructs [104–106] that are both porous and nonporous. Several methods for the production of constructs have been described including solvent casting and carbon dioxide foaming techniques and more

recently electrospinning which is gaining popularity due to the ability to control nanoscale fiber alignment which can be used to improve mechanical strength of the construct and also for topographical functionalization in NGCs [105]. Typical immunological foreign-body response to in vivo implantation occurs with increased macrophage activity around PCL-based conduits [105]. The surrounding macrophages aid the biodegradation process of hydrolysis, although enzymatic degradation has also been demonstrated to cause degradation [106]. Once broken down, complete resorption and excretion appears to be around 135 days in rats, with no evidence of bioaccumulation in internal organs [107].

Biocompatibility of PCL NGCs has been demonstrated in commonly used rat-sciatic nerve injury models with good evidence for peripheral nerve regeneration across short nerve gaps up to 15 mm [102,103]. In humans PCL has been blended with PLA into a biocompatible copolymer poly [65/35(85/15 L/D) lactide *ε*-caprolactone] phospho-ester which is currently available clinically, called Neurolac [Polyganics, FDA 510(k) 2003; CE mark 2004]. Extensive in vivo preclinical data has been described for the use of Neurolac and in randomized clinical trials it has been described as having comparable efficacy to autografts in defects up to 20 mm [108]. Despite the evidence of comparable effect to nerve autograft in randomized trials; clinical experience with the use of Neurolac has demonstrated some mixed outcomes in the adequacy of nerve regeneration in digital and wrist sensory nerve gap injuries up to 25 mm in length [109]. Adverse events such as transitory local irritation and delayed wound healing have been reported [108].

23.7.2 Polyurethanes

Polyurethane (PUR) elastomers (combining both aliphatic hard and aliphatic polyester or polyether elastic components) generally offer excellent modifiable mechanical properties for use as a NGC in addition to good biocompatibility [110]. They can be formed through a variety of techniques such as salt leaching, electrospinning, and carbon dioxide foaming and similarly to PCL, they are biodegraded through a process of hydrolysis [111]. Biodegradable synthetic PURs offer a range of potentially modifiable mechanical, biological, and physical properties depending on the selection of intermediates. Combined with collagen, they have demonstrated good biocompatibility and regenerative outcomes across short (7 mm) rat peroneal nerve gaps [112] and similarly when combined with poly(*ε*-caprolactone) in 10 mm rat-sciatic nerve gap injuries [113].

Their use clinically has been limited in part be due to concerns surrounding the degradation products of biostable implants designed not to degrade such as breast implants. PUR foam that has in the past been used to cover certain breast implants has demonstrated toxic and carcinogenic degradation products [114], but it is unclear whether these degradation products can reach physiologically significant levels in vivo [115] and there has been no evidence of these toxic degradation products in biodegradable PURs used in the production of NGCs.

23.7.3 Polyglycolic acid

PGA conduits, although being the first synthetic degradable NGCs, have demonstrated efficacy in animal models with gap defects up to 30 mm and similar efficacy to primary

repair or nerve graft repair in a randomized clinical trial of small (<8 mm) nerve gap injuries [116]. There is preclinical and clinical safety and efficacy data for Neurotube [116,117] [Synovis, CE mark 1995; FDA 510(k) 1999] but in larger digital nerve gap injuries (≤ 40 mm) Neurotube did not perform as well as the synthetic NGC market leader Neurolac due to its rapid degradation and reduction in mechanical strength which has limited its use [60,118].

23.8 Summary

Peripheral nerve injury can lead to a significant functional deficit especially in more proximal injuries of the brachial plexus in the upper arm. Treatment remains purely surgical with direct neurorrhaphy the preferred method of repair in injuries where no gap between the nerve ends exists (usually <5 mm). Where a gap does exist and direct repair would create tension at the repair site, which is known to lead to poorer outcomes [14], the peripheral nerve autograft remains the gold standard method of treatment. Harvesting of a peripheral nerve autograft creates a second operative site, a secondary nerve injury, and is limited in supply. For these reasons an alternative to the nerve autograft has been sought that can offer similar regenerative capabilities (Fig. 23.2).

Alternative autologous materials provide an obvious solution with reverse vein grafts offering an abundant resource of laminin-rich tubular structures with minimal functional loss left after their harvesting. Comparable results to nerve autograft appear achievable over short nerve gaps (<30 mm), however the risk of collapse precludes their use in long nerve gap repairs. Processed nerve allograft removes the need for a secondary operative site and offers comparable results to nerve autografts over short nerve gaps (<3 cm), and Type I bovine collagen conduits provide another alternative. These have been approved for clinical use in both the United States and Europe, however their cost is still prohibitively high for widespread clinical use and they still fail to address some of the key neurobiological factors that the peripheral nerve autograft offers [119].

Focus amongst peripheral nerve researchers has thus shifted to synthetic materials that are relatively inexpensive compared to biological compounds and can be engineered with modifiable physical and mechanical characteristics such as strength, permeability, and degradation rate as well as cell attachment and proliferation [101]. Synthetic polymers appear to display these characteristics and appear to provide the most suitable NGC material especially when blended with synthetic proteins such as gelatin, fibrin, keratin, and silk or other compounds such as chitosan in order to create 3D constructs and scaffolds that allow for seeding of neurotrophic factors and cells [118]. Improvement in these constructs to allow for better 3D cell seeding and survival of cells in vivo remains a challenge but will likely hold the key to improving peripheral nerve regeneration over longer nerve gaps and in larger mixed (motor and sensory) nerves.

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Cardiac responses to biomaterials

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24.1 Biomaterials for cardiac applications

Biomaterials are natural or synthetic materials that can be introduced into the body to augment or repair part of an organ system. The use of biomaterials to repair organ systems can be traced back to ancient Egypt where a hammer head was used to repair damaged hips [1]. One of the essential requirements for biomaterials is that they are not toxic. In this case the definition of nontoxic includes noncarcinogenic, nonpyrogenic, nonallergenic, blood compatible, and non-inflammatory [1]. In this chapter we will focus on the immune reaction to biomaterials, the foreign body response (FBR). In particular, we will focus on the FBR to biomaterials which have been used for cardiac applications. First, we will review the FBR generally before going on to outline the main biomaterials used for cardiac applications and the FBR to these. Finally, approaches to inhibit or mitigate the FBR will be discussed.

24.2 Foreign body response

Any material, other than those produced by the body itself, can generate a FBR. The intensity of this response depends on the material involved and the anatomical site [1]. The overall physiological response to a biomaterial involves the initial inflammatory response to

biomaterial implantation (inflammation and wound healing), the FBR, and fibrous encapsulation of the material. If the surface cues on, or material released from the biomaterial are recognized as “foreign” by the immune system, the FBR ensues. As shown in Fig. 24.1, the FBR begins with protein adsorption onto the biomaterial [2,3]. The amount and type of proteins that adsorb onto the material will depend on any surface charge on the material and its hydrophobicity [2]. In response to these adsorbed proteins large numbers of neutrophils and macrophages will be attracted to the area immediately surrounding the biomaterial. Adherence of macrophages, derived from circulating monocytes, to the implant surface then occurs as they attempt to remove the foreign body [4,5]. Macrophages are normally the most abundant cell type surrounding a biomaterial implant, primarily because they survive for longer than neutrophils, and so characterize a more chronic inflammatory response [5]. When the macrophages cannot phagocytose the implant, fusion of macrophages occurs to form multinucleated foreign body giant cells [1,6].

Giant cells formed by fusion of macrophages express cytokines, particularly TGF- β , and chemokines which further propagate the FBR, leading to the recruitment of fibroblasts to

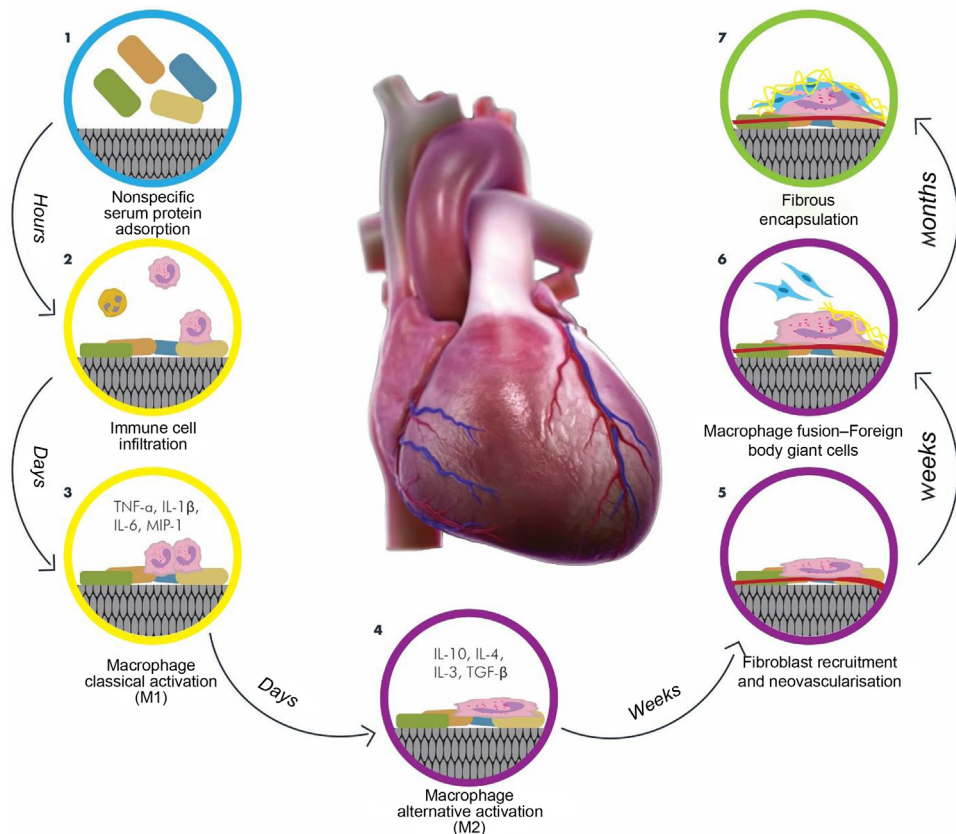


FIGURE 24.1 Initiation and progression of the foreign body response to a biomaterial.

the implant site [4,6]. TGF- β , in particular, causes the activation of myofibroblasts around the biomaterial. These myofibroblasts can secrete procollagen, thus initiating the development of a fibrous capsule around the biomaterial [4]. Fibrinogen also contributes to formation of this fibrous capsule [4].

Formation of a fibrous capsule around the biomaterial implant may affect implant performance. Sharkawy et al. determined that the diffusion time of glucose from stainless steel or polyvinyl alcohol “cages” implanted in mice was increased from 5 to 20 minutes in the presence of a fibrous capsule [7]. In our own group we have observed the development of a fibrous capsule around a thermoplastic polyurethane (TPU)/polycarbonate device attached to the external surface of the rat heart wall. Diffusion of dextran with a molecular weight of 10 or 40 kDa through the fibrous capsule was investigated *ex vivo* 20 days following device implantation. Diffusion of both 10 and 40 kDa dextran through the capsule remained possible at this time although diffusion of 10 kDa dextran was five times faster than its 40 kDa counterpart. This suggests that the effect of the fibrous capsule on diffusion increases with increasing molecular weight of the diffusing compound [8]. As well as affecting diffusion of molecules out of a device, the capsule formed because of the FBR may affect transport of molecules into the implant. This is important in the case of cell-loaded biomaterials where the cells rely on transport of nutrients into the biomaterial [6,9,10]. While angiogenesis may occur through the fibrotic area, there is likely to be an overall reduction in nutrient transport to the cells.

The FBR may inhibit integration of the biomaterial with the host tissue. This phenomenon is observed with silicone breast implants used for reconstruction following mastectomy. The implants do not integrate with host tissue and consequently can cause pain, capsular contraction, and ultimately the requirement for further surgery [11]. Thus, inadequate integration with the host tissue can cause both patient morbidity and implant failure [12]. Therefore it is imperative to investigate the FBR to all materials considered for biomedical applications.

24.3 Biocompatibility testing of biomaterials

The most basic requirement for any material for biological implantation is that it is not toxic. The ISO have published guideline 10993-5 for toxicity testing of materials and medical devices for human use [13–15]. These guidelines stipulate that the material being investigated should not reduce cell metabolic activity *in vitro* to less than 70% that of untreated cells [13]. Other tests commonly performed in early stage *in vitro* biocompatibility testing include staining of cells with fluorescent dyes which are activated by intracellular esterases, bound to specific sections of the cell or travel through damaged cell membranes. This staining allows assessment of cell morphology and complements metabolic activity assays by giving qualitative and quantitative information on cell viability [15]. Hemocompatibility testing is also performed using lysis of red blood cells as a marker of biocompatibility [15].

Following *in vitro* assessment, *in vivo* testing in animal models is performed. The European Medicines Agency (EMA) has published guidelines for the preclinical toxicity testing of newly developed medicinal products [16]. Among the EMA recommendations are examination of the effects of repeated dosing and potential genotoxicity, carcinogenicity, and reproductive toxicity. Immunotoxicity is also mentioned in the EMA

guidelines and is mandatory when the medicinal product shows signs of immunosuppression or enhancement. Furthermore, the EMA require chronic toxicity testing for at least 6 months in an animal model before an application to carry out a clinical trial will be considered [16]. Generally, assessment of the FBR occurs as part of this suite of *in vivo* tests. Information on the FBR is not always recorded or reported for *in vivo* studies. Where the FBR is reported, the level of detail can vary greatly from simply stating that a fibrous capsule containing foreign body giant cells was present, to histological quantification of the tissue or proteomic or genomic assessments.

24.3.1 Identification and quantification of the foreign body response—histology

Myofibroblasts at the implant site secrete procollagen, resulting in a high collagen content in the fibrous capsule surrounding biomaterial implants. Polarized light microscopy can be used to assess the structure of the fibrous capsule in terms of fiber organization and to distinguish between immature and mature collagen fibers [17]. Masson's trichome staining can also be used to quantify collagen in the tissue surrounding the implant [17,18]. Histology of the tissue surrounding a biomaterial implant allows identification of differences in cell concentrations in the tissue adjacent to the implant. Immunofluorescence staining for α smooth muscle actin can be used to quantify the amount of myofibroblasts present. Toluidine blue stain is used to identify mast cells. F4/80 staining identifies all macrophages, while CD206 (M2 macrophage marker) and CCR7 (M1 macrophage marker) can be used to ascertain the relative amounts of M1-like and M2-like macrophages [18,19]. This is important, as M1 macrophages are proinflammatory and will assist in propagating the FBR, while M2 macrophages are anti-inflammatory and contribute to constructive tissue repair [19].

Even in the absence of gross fibrous capsule formation, the concentration of immune system cells may be increased in the tissue surrounding the implant, raising questions about the long term biocompatibility of the implant [5,12]. Integration of a biomaterial with the host tissue is generally impeded by the FBR and so infiltration of host cells into a biomaterial may indicate that it has not been subjected to the FBR [12]. In particular, CD31 staining can be used to identify endothelial cells in the biomaterial implant indicating the initial steps in vascularization of the implant and thus tissue integration [17]. The importance of integration with host tissue has been identified in our own group in a study of implants on the external surface of a rodent heart wall. Efficient coupling of soft robotic cardiac assist devices to the external surface of the heart is crucial to augment cardiac function and represents a hurdle to translation of this technology. In this study we sought to take advantage of the host response to mechanically couple an epicardially placed soft robotic sleeve to the myocardium whereby two sleeve material candidates were compared. Silicone was compared to "medical mesh" [a poly(ester) mesh coated with collagen, poly(ethylene glycol) (PEG) and glycerol], and the FBR was measured 14 days postimplantation via histology and measurement of the fibrous capsule. The impervious nature of the silicone impeded cell infiltration thus preventing integration with the host tissue. This resulted in complete fibrous encapsulation of the silicone implant. The medical mesh, which permitted cellular infiltration, successfully integrated and coupled with the host tissue [20].

24.3.2 Identification and quantification of the foreign body response—proteomics

Protein adsorption onto the biomaterial is one of the first steps in the FBR. Proteomics can identify adsorbed proteins leading to a better understanding of the underlying mechanism of the FBR. Chromatography or mass spectrometry can be used to identify the proteins. Analysis of the proteins adsorbed onto PEG–arginine-glycine-aspartate (RGD) and PEG–arginine-aspartate-glycine (RDG) hydrogels over 28 days incubation in mice showed that out of the 300 proteins attached most were those traditionally associated with wounds or acute inflammation [21]. Furthermore, the top 90% of proteins that were identified were present in all three groups, PEG alone, PEG–RGD, and PEG–RDG [21]. Albumin was the protein most commonly identified followed by Apolipoprotein A-1 and Carbonic anhydrase 3 [21].

24.4 Biomaterials

Biomaterials can be classified into four broad categories: polymeric biomaterials, metals, composite materials, and ceramics. Polymeric biomaterials are the most common type of materials utilized for cardiac applications. Mechanical properties of synthetic polymeric biomaterials, for example, acrylics, poly(ester)s, and poly(urethane)s (PUs) can easily be modified to suit the application, for example, mechanical strength for tissue support or low viscosity for catheter delivery. However, such synthetic polymers may exhibit poor biocompatibility and prompt inflammatory reactions [1]. Metals are used in pacemakers, stents, and in the manufacture of artificial hearts. While generally considered biocompatible, chemical reactions may occur with ionized metals *in vivo* leading to degradation or a change in the surface properties of the material [1]. Composite materials are a combination of biomaterials. The overall properties of composite biomaterials are dependent on the individual constituents and their interaction with each other. However, their more complex composition makes the immune response to these materials more difficult to predict. Ceramics have been used for the design of prosthetic heart valves, but their poor tensile strength means that they are less favorable than polymeric biomaterials or metals for this application [1].

Biomaterial selection depends on the biomedical application being investigated. Biomaterials' use for cardiac applications can be divided into four categories: mechanical support, cell delivery, drug delivery, and implantable devices and both the material used and the application can influence the FBR [22]. Depending on the application and the mechanical properties of the biomaterial, several routes of administration are available for cardiac delivery. The potential administration routes are shown in Fig. 24.2. The various biomaterials used for cardiac applications have been reviewed recently by our group [22]. Here we will review biomaterials based on the applications for which they are most frequently used. However, most biomaterials have been investigated for multiple applications. In some cases, the specific cardiac FBR to a particular material may not be available in the literature. In this case, the FBR reported for the biomaterial in other biological tissues will be outlined.

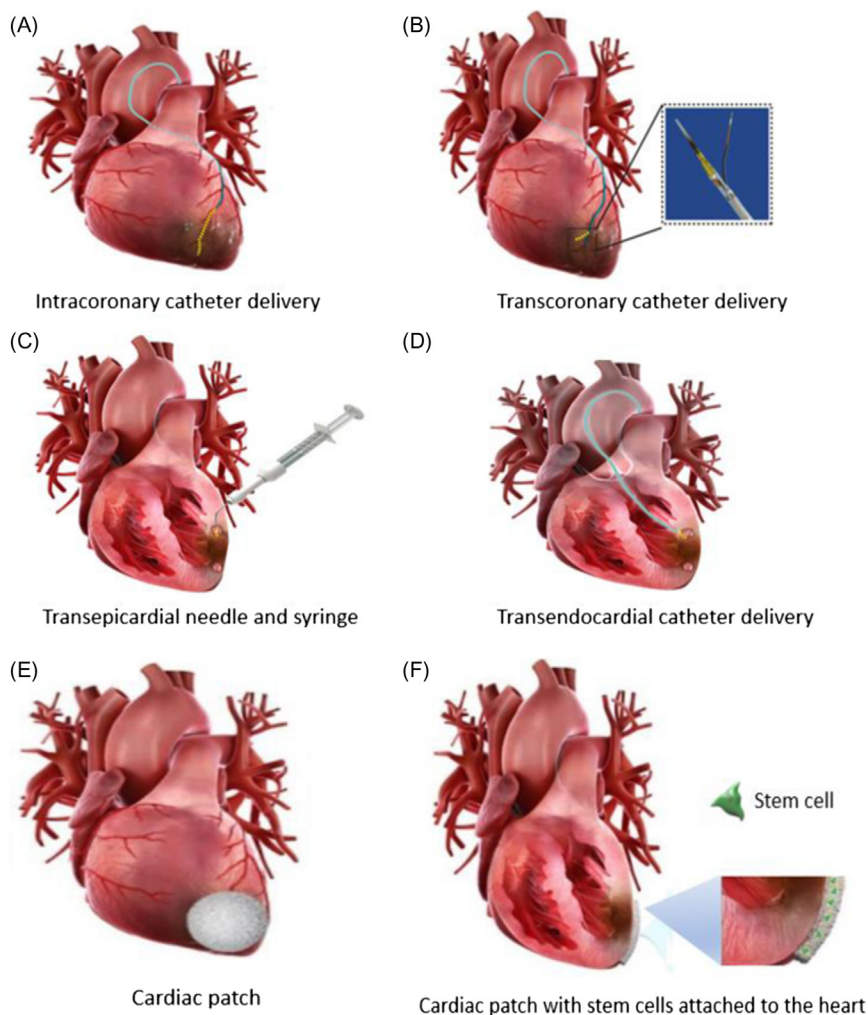


FIGURE 24.2 Routes of administration used to deliver various types of biomaterials to the heart. (A) Intracoronary delivery into the coronary arteries. (B) Transcoronary delivery through the coronary vasculature into cardiac tissue. (C) Transepicardial delivery into cardiac tissue from the external surface of the heart. (D) Transendocardial delivery into the cardiac tissue from the inside the heart. (E) Cardiac patch attached to the external surface of the ventricle. (F) Stem cells in a cardiac patch in contact with cardiac tissue.

24.4.1 Mechanical support

Materials may be injected into the ventricle wall to provide structural support to the weakened wall after myocardial infarction (MI) and to increase the ejection fraction. Alginate hydrogels, Algisyl® and IK-5001 and an extracellular matrix (ECM)-based hydrogel, VentriGel, have progressed to clinical trials for this application. Other biomaterials which have been used

include hyaluronic acid (HA), chitosan, collagen, fibrin, Matrigel®, keratin, calcium hydroxyapatite, and peptide or PEG hydrogel systems [22].

24.4.1.1 Alginate

Sodium alginate is possibly the most promising biomaterial reported to date for increasing ventricular wall strength and improving ejection fraction [22–24]. Alginate has FDA approval as a food additive [25]. Results from alginate gels in animal studies led to the first clinical trials of alginate gels (Algisyl and IK-5001) in humans [26]. IK-5001 is an alginate gel, which is delivered via the affected coronary artery using a percutaneous technique and forms a stiff implant in areas of high calcium concentration such as at the point of infarct [27]. Algisyl is administered via limited thoracotomy to the wall of the left ventricle where it gels, increasing the strength of the ventricle wall [28]. In our own group, we have developed an alginate/decellularized porcine ECM hydrogel which can be injected through a single-lumen catheter potentially allowing minimally invasive delivery. The biocompatibility of this alginate/ECM hydrogel was demonstrated *in vitro* by its ability to support the survival of dermal fibroblasts [29]. We have also demonstrated that an alginate hydrogel can provide an 8-fold improvement in retention of mesenchymal stem cells (MSCs) in a rat heart while an alginate epicardial patch improved MSC retention 59-fold compared to injection of MSCs in saline [30].

No information is currently available on the FBR to Algisyl or IK-5001. However, alginate has previously been noted to activate the FBR *in vivo* in both rodents and nonhuman primates. In humans, the alginate/calcium combination hemostatic dressing Kaltostat has been shown to induce an FBR if fibers are left *in situ* for 7 months [31]. However, strategies to mitigate the FBR to alginate may be possible. Veis et al. have reported that, in the case of alginate microspheres loaded with pancreatic islets for the treatment of type 1 diabetes, adjusting the size of the microspheres from 0.5 to 1.5 mm can improve the biocompatibility of the microspheres in both rodents and nonhuman primates [32]. Vegas et al. have reported that chemical modification of alginate with a triazole group can reduce the FBR in mice, an approach which may be of use for cardiac applications [6]. Balakrishnan et al. investigated the use of an alginate–gelatin–borax hydrogel in a rat wound model and found that while the FBR was evident 7 days after gel application, the FBR appeared to have abated at 15 days. They suggested that this disappearance of the FBR was due to the degradation of the alginate–gelatin–borax hydrogel and indicated that the degradation products of the hydrogels were not immunogenic [2].

24.4.1.2 Decellularized tissue

VentriGel, a hydrogel formed from decellularized porcine myocardial tissue is currently in phase 1 clinical trials in patients between 60 days and 3 years post MI. VentriGel is being delivered to the ventricle percutaneously using a MyoStar catheter [33]. Preclinical studies of VentriGel in a rodent model of MI did not indicate the presence of foreign body giant cells surrounding the VentriGel 2 weeks following implantation [34]. However, other, similar materials such as bovine pericardial scaffolds crosslinked with 0.6% glutaraldehyde (XenoLogiX™ and PeriGuard®) have caused the development of an FBR which was present 12 weeks following subcutaneous implantation in a rat model [35].

Within our own group we have used decellularized porcine tissue for the creation of vascular scaffolds [36]. Such scaffolds commonly displayed poor recellularization and we have produced significant innovative strategies to improve recellularization of these decellularized tissues thus improving their potential for translation to the clinic. We have determined optimal scaffold porosities, mechanical properties, freeze–drying procedures, and recellularization procedure [36,37], and, in unpublished work, have incorporated particulate systems delivering therapeutics to control the immune response.

24.4.1.3 Hyaluronic acid

HA hydrogels have been used for mechanical support of the ventricle wall as well as for drug and cell delivery. HA is an ECM component produced by cells of mesenchymal origin and is involved in multiple physiological processes including angiogenesis [38]. The suitability of HA as a biomaterial for human use has been established during its 30 years of clinical usage as a cosmetic product, an artificial tear substitute and as an intra-articular injection for osteoarthritis [39,40]. In the cosmeceutical field the HA dermal filler Hylaform®, has been reported to cause an FBR, but is licensed for human use [41].

In addition to native HA, many chemically modified HA derivatives have been synthesized which have better mechanical properties than the parent molecule. This increases the range of biomedical applications for which HA could be utilized [42]. However, chemical modification may alter the FBR to HA and this should be considered in biomaterial design and development.

Within our own group, we have performed extensive work on HA hydrogels for cardiac applications. The HA molecules used are chemically modified with tyramine or hydroxyphenyl to facilitate crosslinking and hydrogel formation in the presence of the relevant crosslinkers, hydrogen peroxide (H_2O_2) and horseradish peroxidase. HA molecules modified with the RGD peptide sequence have also been used by our group to facilitate cell attachment within the hydrogel. Minimally invasive endocardial delivery of these HA hydrogels can be achieved using the *Advanced Materials Catheter* (AMCath) developed by our own group [43,44]. This dual lumen catheter facilitates the injection of HA dispersions, keeping crosslinkers apart throughout the length of the catheter until they reach a mixing reservoir at the point of injection which allows mixing of HA and crosslinkers facilitating rapid hydrogel formation in the seconds following injection into the heart tissue [44]. *In vitro* the viability of cardiopoietic adipose-derived stem cells (ADSCs) injected through AMCath in a HA hydrogel was 80% compared to that of cells which did not pass through the catheter. *In vivo* AMCath facilitated endocardial delivery of a HA hydrogel to porcine hearts with subsequent retention of the hydrogel in the ventricle wall [44]. These modified HA hydrogels have also been investigated for their potential to deliver growth factors relevant to angiogenesis offering the potential for cell-free therapies for cardiac repair [45]. Thus the HA-based therapeutic systems developed by our group so far can act as a platform technology for delivery of various combinations of therapeutics relevant to cardiac tissue repair and regeneration.

Other groups have also extensively investigated HA-based materials for cardiac applications. Yoon et al. injected an acrylated HA hydrogel into a rat model of MI. Four weeks after implantation, histological evaluation of the tissues surrounding the injected hydrogel showed no inflammatory cells in the region of the injected hydrogel [46]. Eight weeks

following implantation of an adamantane-modified HA hydrogel into an ovine heart Rodell et al. reported minimal foreign body giant cells in the vicinity of the implanted hydrogel [47]. Chen et al. also injected an adamantane-modified HA hydrogel into the left ventricle of rats immediately following induction of MI in the rat. One week following hydrogel injection, the acute immune response was measured using CD68⁺ staining and exhibited that inclusion of IL-10 in the HA hydrogels reduced the number of macrophages surrounding the hydrogel [48]. This suggests that both the implanted biomaterial and any loaded cargo can influence the FBR.

Fiumana et al. observed an FBR to their implanted hyaluronan-based HYAFF11 scaffold loaded with MSCs 4 weeks following implantation in a rat heart grafted in the abdomen of another rat [49]. The FBR was again noted with the HYAFF11/MSC system in a porcine model of MI, however the FBR was noted to be confined to the hyaluronan fibers and the overall number of immune cells (lymphocytes) in the region surrounding the scaffold was not significantly different to that in the untreated control groups [50].

Timing of analysis might also impact the FBR observed. Kim et al. formulated HA hydrogels by crosslinking HA microbeads with divinyl sulfone. While some acute inflammation was noted around the hydrogels 4 weeks following intradermal implantation, this response had subsided at 8 weeks [51]. No fibrous capsule was formed around the hydrogel indicating that any FBR abated before capsule formation occurred. A similar response was noted by Tian et al. in response to implantation of a HA–poly-D-lysine hydrogel in a rat brain where foreign body giant cells were observed at the implantation site at 6 weeks, but not observed thereafter. The HA–poly-D-lysine hydrogel integrated with the host tissue, leading the authors to conclude that the hydrogel was inert in terms of immune response [52].

24.4.1.4 Synthetic biomaterials

Polytetrafluoroethylene (PTFE) is used preclinically for the creation of vascular grafts. In an *in vivo* study in a mouse model, PTFE implanted subcutaneously caused a less severe FBR than other biomaterials such as poly(vinyl alcohol) and Prolene [poly(propylene)] [18].

Fujimoto et al. have previously compared poly(ester urethane urea) (PEUU) scaffolds to expanded PTFE for cardiac reconstruction of adult rats [53]. While both scaffold types were surrounded by a fibrous capsule at all time points, 4, 8, and 12 weeks, cellular ingrowth and integration with the host tissue was only evident in the PEUU group. Furthermore, macrophage infiltration in the PEUU group was less than in the PTFE group. This ability of the PEUU to integrate with the host tissue may prevent complications, such as chronic inflammation and implant failure associated with the FBR.

Yoshizumi et al. have reported on the injection of a poly(NIPAAm-*co*-HEMA-*co*-MAPLA) [where NIPAAm = *N*-isopropylacrylamide, HEMA = 2-hydroxyethyl methacrylate, and MAPLA = methacrylate-poly(lactide)] into rat hearts, immediately, 3 days or 2 weeks after MI. While the authors identified the optimal injection time for functional improvement (3 days post-MI), histological examination of the tissue showed that foreign body giant cells were present when samples were excised 10 weeks after implantation regardless of the timing of injection post MI [54].

24.4.2 Cell delivery

Delivery of cells to the heart is of interest for tissue engineering (TE) and regenerative medicine applications. Cells may have a regenerative effect on the heart by one of two means, either the delivered cells may affect tissue repair themselves or they may secrete paracrine factors which leads to activation of resident cells and consequently tissue repair or regeneration [55]. Cells can be delivered to the heart in saline or in a hydrogel or polymeric matrix system using materials such as HA or alginate which were reviewed in the last section. Implantable devices and patches have also been used for cell delivery.

24.4.2.1 *Fibrin*

The first study using a hydrogel to deliver skeletal myoblasts to a rat heart used a fibrin hydrogel [56]. Histology was performed 5 weeks following implantation of the cell-laden hydrogel and no FBR was reported [56]. Indeed fibrin is widely cited as being free of any associated FBR and thus potentially being the ideal biomaterial for cardiac applications [57]. Zhang et al. attempted to take advantage of this good biocompatibility of fibrin by formulating a PEG–fibrin hydrogel to deliver bone marrow-derived mononuclear cells and the growth factor HGF (hepatocyte growth factor) to the heart in a mouse model of MI [58]. Zhang et al. did not report the FBR to this material, but such use of composite materials may complicate the FBR.

24.4.2.2 *Poly(ethylene glycol)*

PEG hydrogels are known to elicit a FBR. This is due to PEG being susceptible to macrophage degradation and protein adsorption onto PEG hydrogels [59]. The FBR to PEG hydrogels begins within 2 days of implantation and persists beyond 4 weeks and is thought to be a result of the hydrophobicity of the polyacrylate chains crosslinked by PEG in the most commonly used PEG–diacrylate polymers [59]. Injection of a nondegradable PEG hydrogel into a rat ventricle resulted in an FBR evident at 3 months post hydrogel injection. Late adverse remodeling was also evident at 3 months following hydrogel injection, although the contribution of the FBR to this process was unclear [60].

Strategies to reduce the FBR to PEG-based materials have been investigated. Addition of MSCs to a PEG hydrogel implanted subcutaneously in mice reduced fibrous capsule thickness compared to implantation of unloaded PEG hydrogels indicating that the immunomodulatory effects of the MSCs may influence the extent of the FBR [61]. Reduction of the FBR to a PEG–RGD hydrogel was observed on reducing the Young's modulus from 840 to 130 kPa [62]. A similar trend was observed by Jansen et al. where reducing the Young's modulus and zwitterionic character of PEG–phosphorylcholine hydrogels implanted subcutaneously in mice reduced the FBR [58]. Thus, both the chemical composition and the mechanical properties of PEG hydrogels can affect the FBR.

24.4.2.3 *Cardiac patches—poly(ester urethane)*

Cardiac patches can also be used for cell delivery to the epicardial surface. Cardiac patches can be attached to the outer surface of the ventricle wall from where they can deliver loaded therapeutics [63]. Our group has developed a poly(ester urethane) (PEU) and PEUU-based bioresorbable cardiac patch for mechanical stabilization and delivery of

therapeutics to the epicardial heart wall [17]. This patch, referred to as SPREADS—Surface PRone EpicAr dial Delivery System has a semipermeable membrane on the side attached to the heart wall to facilitate diffusion of therapeutics into the heart tissue. The patch has a reservoir in the middle, between the PEU and PEUU layers to accommodate the desired biomaterial. SPREADS also has a bioadhesive reservoir to facilitate attachment to the heart. In our initial studies of the SPREADS system we have loaded it with an RGD functionalized HA hydrogel containing cardiopoietic ADSCs and tested it in a porcine model of MI. 28 days following attachment of SPREADS a significant improvement in left ventricular ejection fraction compared to the gold standard treatment which was achieved in groups treated with SPREADS loaded with HA gel alone or a HA gel loaded with ADSCs. This SPREADS device could easily be used to deliver any other combination of biomaterials and therapeutic agents [17].

24.4.2.4 Cardiac patches—polycaprolactone

A patch composed of self-assembling peptides and reinforced with polycaprolactone (PCL) has been granted a patent in both Europe and Japan. This patch claims to be biocompatible and biodegradable and is intended to provide structural support to the heart wall; however, results from clinical trials on this patch have yet to be published [64]. Other PCL-based materials including a PCL–gelatin patch have previously been reported for cell delivery to the heart and improvement of cardiac function. Wang et al. used a PCL–gelatin patch on the surface of the heart to deliver MSCs but did not report on the associated immune response [65].

PCL has been reported to elicit the FBR, with topography of the PCL scaffold likely to affect the extent of the FBR. *In vitro* PCL scaffolds with a random fiber orientation have been shown to have a higher density of attached monocytes and subsequently macrophages than PCL scaffolds with an aligned fiber orientation. This trend was replicated *in vivo* where PCL scaffolds with random fiber orientations elicited a more intense FBR than those with aligned fibers when implanted subcutaneously in a rat [66]. Thus, similar to the other biomaterials discussed herein, the FBR to PCL depends on the chemical composition and the discrete spatial arrangement of the material.

24.4.2.5 Cardiac patches—collagen

Collagen, being a major component of bone has been widely investigated within our own group and others for its potential applications in bone tissue engineering [67,68]. Collagen has also been assessed for its potential as a cell-loaded cardiac patch. In our group O'Neill et al. investigated the use of sustained release HGF and insulin-like growth factor 1 (IGF-1) alginate-based microparticles in a collagen patch to promote proliferation of stem cells *in vitro* [69]. We have also shown that MSC retention in the rat heart is increased 47-fold with the use of a collagen patch compared to injection of MSCs in saline [30].

Arana et al. used a collagen patch to deliver ADSCs to the epicardial surface of the heart in both rats and mini pigs. This collagen/ADSC patch resulted in a functional improvement in porcine hearts compared to untreated pigs. However, the FBR to the formulation was not reported [70]. A vascular endothelial growth factor (VEGF)-loaded collagen patch improved vascularization and wall thickness in a rat model of MI at 7 days, but

again no information was provided on the FBR [71]. Ye et al. studied the effect of collagen crosslinking mechanism on the FBR for subcutaneously implanted collagen scaffolds in mice. Collagen scaffolds, formed from sheep collagen crosslinked with glutaraldehyde, promoted infiltration of neutrophils resulting in complete degradation of the scaffold by day 28. Collagen scaffolds crosslinked with hexamethylenediisocyanate (HDSC) remained intact at day 28 although foreign body giant cells were present [72]. Furthermore, macrophages surrounding HDSC crosslinked collagen scaffolds did not express markers of either M1, classically activated macrophages or M2, alternatively activated macrophages, but were an “FBR-specific phenotype” [73]. This indicates, as we have seen previously in this chapter, that the source of the biomaterial and chemical nature of the crosslinking can affect the FBR.

24.4.2.6 Cardiac patches—poly(urethane)

Within our group, we have developed a thermoplastic PU/poly(carbonate) replenishable reservoir device which has been used preclinically to deliver bone marrow-derived MSCs to a mouse heart. Twenty days following implantation a fibrous capsule had formed around the device. Diffusion testing through this fibrous capsule *ex vivo* found that dextran could diffuse through the fibrous capsule with more rapid diffusion occurring with 10 kDa dextran compared to 40 kDa dextran [8]. Thus while fibrous capsule formation reduced the rate of diffusion, the dextran molecules were still capable of traveling through the fibrous capsule. Thus, in considering the effect of the fibrous capsule on delivery of therapeutics one must consider the factors that affect diffusion, the molecular weight of the therapeutic, the thickness of the fibrous capsule, and the local concentration gradient. We have recently developed a scaled up regenerative reservoir platform (Regenervoir) for use in large animal models, with relevance to cardiac, abdominal, and soft tissue pathologies. Regenervoir incorporates multiple novel design features essential for clinical translation, with a focus on scalability, mechanism of delivery, fixation to target tissue, and filling/refilling with a therapeutic cargo, and is demonstrated in an array of clinical applications that are easily translated to human studies. Regenervoir consists of a porous reservoir fabricated from a single material, TPU, capable of delivering cargo via fill lines to target tissues. Cardiac-Regenervoir was designed specifically for congestive heart failure and is composed of a multidepot reservoir that is wrapped around the left ventricle in a minimally invasive manner to allow focal delivery of therapeutic cargo. While we have investigated the immune response to TPU devices in small animals previously, the next steps in the development of Regenervoir will focus on determining the immune response in large animal models [74,75].

24.4.3 Growth factor/small molecule delivery

Growth factors or small molecules may be delivered to the heart using the polymeric matrices discussed in the previous section, however, to achieve an appropriate release profile for these loaded therapeutics, a second matrix such as a microparticle or nanoparticle may be needed. These particulate systems can be synthesized using either natural or synthetic biomaterials as carriers [25,76]. Initially, nonbiodegradable materials were employed for particle fabrication. These included poly(methyl methacrylate), poly

(acrylamide), and poly(styrene)s, however signs of chronic toxicity with these agents required a movement toward more biocompatible materials [77]. While natural materials are advantageous in terms of their biocompatibility and degradation, controlled release and quality control may be difficult [25]. The materials employed to date for achieving sustained drug delivery to the myocardium, either by formation of a hydrogel scaffold or as particulate systems, are alginate, poly(lactide-co-glycolide), poly(lactide-co-glycolic acid) (PLGA), NIPAAm, chitosan, and HA. Alginate and HA have been discussed previously in this chapter, the FBR to chitosan, PLGA, and NIPAAm will be reviewed in this section.

24.4.3.1 Chitosan

Chitosan, the second most common polysaccharide found in nature, is obtained from the exoskeleton of crustaceans and is biocompatible, biodegradable, and antibacterial [25]. Thermoresponsive chitosan hydrogels, which are liquid at room temperature, but form a 3D hydrogel network at 37°C, have been used for cell and growth factor delivery to the heart. Our group previously developed a chitosan/ β -glycerophosphate thermoresponsive hydrogel that forms a gel at 33°C [78]. This hydrogel facilitated incorporation of MSCs and the proangiogenic chelating agent desferrioxamine. Although this hydrogel was initially developed for applications in critical limb ischemia, the principle of promoting angiogenesis is relevant in the ischemic myocardium post MI [78]. We have also shown that MSC retention in the rat heart is improved 14-fold when MSCs are injected in a chitosan/ β -glycerophosphate hydrogel compared to injection in saline [30].

Wang et al. used a thermoresponsive chitosan hydrogel to deliver basic fibroblast growth factor (bFGF) to the rat heart following induction of MI. Injection of chitosan + bFGF significantly reduced infarct size and fibrotic area and increased left ventricular ejection fraction compared to injection of bFGF in saline [79]. No information on the FBR was reported [79]. A thermoresponsive chitosan hydrogel has also been used to deliver embryonic stem cells (ESCs) to a rat heart following induction of MI. Injection of chitosan and ESCs improved wall thickness and microvessel density more than injection of saline alone [80]. Again, the FBR to the hydrogel was not reported, but the potential of chitosan hydrogels for cardiac regeneration is clear. Subcutaneous or intraperitoneal implantation of chitosan hydrogels in rats elicited an FBR, however, this was less severe than the FBR induced by Vicril (polyglactin 910) sutures which are currently licensed for human use [81].

It has been established that the chemical structure of chitosan can impact the FBR. Chitosan with a degree of acetylation (DA) of 5% attracted fewer inflammatory cells following implantation in a rodent air-pouch model than chitosan with a DA of 15%. The macrophages surrounding the chitosan with a DA of 5% were mainly M2 alternatively polarized macrophages which induced a benign anti-inflammatory response whereas the 15% DA chitosan attracted more proinflammatory M1 polarized macrophages [19]. Again this indicates that careful consideration of the chemical properties of biomaterials can help to mitigate the FBR.

24.4.3.2 Poly(lactide-co-glycolic acid)

PLGA has FDA approval for multiple indications and is used clinically in sutures as well as in licensed microparticle formulations [25]. PLGA-based microparticles and nanoparticles have been developed for sustained release of growth factors relevant to cardiac TE and have been tested both *in vitro* and *in vivo* [82–84]. In our own group PLGA

microparticles loaded with IGF-1 have been developed for use in cardiac regenerative applications [85]. However, degradation of PLGA produces acidic products which can promote the FBR [86]. PLGA microparticles loaded with bone morphogenic protein 2 (BMP2) for bone regeneration elicited an FBR at their surface which was evident 21 days following implantation into a rat calvarial defect [87].

24.4.3.3 *N-isopropylacrylamide*

Garbern et al. developed a pH responsive poly(*N-isopropylacrylamide-co-propylacrylic acid-co-butyl acrylate*) [p(NIPAAm-*co*-PAA-*co*-BA)] hydrogel for delivery of FGF to the heart. At pH 6.8, in ischemic tissue, the formulation was a liquid allowing FGF release to promote angiogenesis. In healthy tissue, at pH 7.4, the formulation gelled, preventing FGF release where it was not required. Twenty-eight days following injection into the heart in a mouse model, foreign body giant cells were observed around the hydrogel. The authors concluded that macrophages involved in the FBR may have contributed to the improved angiogenesis seen in the groups containing hydrogel not loaded with FGF. However, the authors also noted that a longer study (>28 days) would be required to confirm the immune response to the implanted p(NIPAAm-*co*-PAA-*co*-BA) [88].

24.4.4 Prosthetic valves

Traditional mechanical heart valves are made from titanium and carbon. These are durable, but carry the risk of thrombus formation on the surface of the valve, requiring lifelong treatment of patients with anticoagulants such as warfarin [89]. Tissue-based valves from horse, pig, or other humans, negate the need for lifelong anticoagulation, but are less durable than mechanical valves, lasting only 10–20 years [89]. The FBR is cited as the most common limitation to all heart valve types, it can contribute to thrombus formation and cause calcification and subsequent blockage of adjacent blood vessels [90,91]. CorMatrix, formed from decellularized porcine small intestine, is an FDA approved substance used for cardiac reconstruction in pediatric patients with cardiac valvular or septal defects. Analysis of 12 explanted samples from 11 patients 77–1294 days post CorMatrix implantation, showed foreign body giant cells were present in 8 out of the 12 samples. Fibrosis was noted in the surrounding tissue in all cases. Furthermore, the CorMatrix had not integrated with the native tissue [12]. Synergraft® is a commercially available heart valve replacement product also formed from decellularized ECM. It has been reported that Synergraft® can cause an FBR, which, in severe cases, may cause valve failure and death [92].

24.4.5 Traditional medical devices

24.4.5.1 *Pacemakers and Implantable Cardioverter Defibrillators (ICDs)*

Pacemakers and ICDs are used to regulate heart rhythm and, in the case of ICDs, to “shock” the heart if a heartbeat is not detected within a certain time interval. Both pacemakers and ICDs have attached leads which are placed in or on the heart chambers. The FBR to pacemaker leads, often composed of PU, can cause complete encapsulation of the

leads [93]. This can increase the energy required by the pacemakers to sense changes in the electrical activity of the heart thereby shortening battery life [93]. It has been observed *in vitro* that increasing the flexibility of the pacemaker leads can reduce cell damage and consequently the FBR [93]. Postmortem studies on patients with either a pacemaker or an ICD have shown that the fibrous capsule is thickest around ICD ventricular electrodes and contains foreign body giant cells [94]. This postmortem study also concluded that while the fibrous capsule surrounding steroid-eluting (dexamethasone) pacemaker leads was thinner than surrounding leads which were not eluting a steroid, foreign body giant cells were observed even in the presence of the dexamethasone [94].

24.4.5.2 Stents

Stents are placed in coronary arteries blocked by atherosclerotic plaque to provide a passage for blood to pass through, thus overcoming the blockage. Stents can be manufactured from a range of materials and can be bare metal, drug-eluting, bioabsorbable, or bioresorbable [86]. Restenosis, blockage of the implanted stent, was a major problem with first generation bare metal stents with an incidence between 17% and 41% [95]. The advent of drug-eluting stents has reduced the rate of restenosis to less than 10% [95]. Dexamethasone, sirolimus, and tacrolimus are among the molecules that have been used to reduce stent restenosis [96]. Stent surfaces promote protein adhesion, mentioned earlier as the first step in the FBR [86]. Thus stents can be subject to the FBR. However, dexamethasone-eluting stents have been developed which can reduce both the potential for restenosis and the FBR [86,97].

24.5 State of the art approaches to reduce the foreign body response

Before discussing ways to prevent the FBR, it is important to note that although many biomaterials, including some of those reviewed above, elicit an FBR, this does not totally preclude their use in the clinic. CorMatrix, for example, is used in pediatric patients despite the FBR occurring and a lack of integration with host tissue, because it can still support or replace damaged tissue [12]. Furthermore, while initially it was thought that the FBR should be completely inhibited, more recently it has been identified that certain macrophage polarization states may facilitate tissue repair and that macrophages might increase angiogenesis. Thus, harnessing the potentially beneficial effects of an immune response such as enhanced repair and vascularization has become important [98]. Also the results of preclinical testing in rodents should be interpreted with caution as, in the case of islet transplantation, it is established that alginate microspheres which are not significantly affected by the FBR in rodents are subject to a vigorous FBR in nonhuman primates and humans [99]. The strategies below may provide a means of mitigating the FBR, thus improving the acceptability of biomaterials as medicinal products.

24.5.1 Material properties

As identified previously in this chapter, Fibrin has been suggested as an ideal biomaterial for cardiac TE due to its reported lack of an FBR. However, all of the other materials

identified elicited some form of FBR. Careful consideration of material properties may yield modified biomaterials which have a significantly reduced FBR compared to the parent material. The first step in the FBR is protein adsorption onto the material surface and so chemical modifications of the biomaterial should be considered to attenuate the FBR. Zhou et al. have developed a novel high throughput screening method which facilitates detection of materials with low propensity for protein adsorption. This technique can therefore identify materials that are less likely to elicit an FBR thus potentially proving a useful tool for investigation of chemically modified biomaterials [100]. This technique was employed by Ma et al. to aid in predicting the protein adsorption onto and FBR to a series of poly(β -amino alcohols). When these poly(β -amino alcohols) were formed into microparticles and injected subcutaneously into a mouse, correlation was seen between the screening data and the *in vivo* FBR [101]. Vegas et al. have identified in both rodents and nonhuman primates that triazole derivatives of alginate are associated with a reduced FBR to alginate microspheres [6].

The mechanical properties of the material are also important. Jansen et al. implanted PEG–phosphorylcholine hydrogels subcutaneously in mice and observed that reducing the Young's modulus of hydrogels from 165 to 3 kPa reduced the FBR to the material [61]. This reduction in FBR with reduced Young's modulus was also observed by Blakney et al. where reducing the Young's modulus of PEG–RGD hydrogels, implanted subcutaneously in a mouse, from 840 to 130 kPa, significantly reduced the concentration of macrophages around the hydrogel and consequently the FBR to the hydrogel [64].

24.5.2 Device design

The FBR to a biomaterial is influenced by the material's chemical and physical properties [5]. Flat, smooth implants will generally have a layer of macrophages attached which is one or two cells thick, however rough surfaces such as PTFE sometimes used on the surface of vascular prosthesis will have an attached layer consisting of macrophages and giant cells [5]. The surface:volume ratio of the implant also influences the cellular composition of the fibrous capsule formed due to the FBR. A high surface:volume ratio will result in a higher ratio of macrophages:giant cells than might be observed with a lower surface:volume ratio implant.

In the area of islet cell transplantation, it has been determined that both the size and shape of alginate microspheres encapsulating islets can affect the immune response in mice [32,102]. No FBR was observed 6 months following implantation of islets in spherical alginate microspheres, 1.5 mm in diameter, in mice [32].

Increasing the porosity of poly(vinyl alcohol) cages implanted subcutaneously in rats, reduced capsule density thus allowing faster diffusion of glucose through the capsule compared to the tightly packed avascular capsules surrounding nonporous cages [7].

24.5.3 Coatings

Coating the surface of a biomaterial has been shown to reduce the FBR. Coating glass or PU materials with kininogen reduces neutrophil adherence [4,103]. However, retention

of the coating on the surface of the biomaterial may be challenging with blood plasma previously shown to remove coatings from PU materials [4].

The FBR is activated when the body identifies an object as “foreign”, however, if the material can be modified to have surface properties similar to native tissue, the immune response may be attenuated. Phospholipid coating is based on the theory that the immune system will not attack the phospholipid surface due to the ubiquitous occurrence of phospholipids in the body. The phospholipid, phosphorylcholine, has been used to coat PU vascular grafts with a subsequent reduction in neutrophil binding to the graft [4,104]. CD200, CD47, and CD55 are immunoregulatory receptors expressed on the surface of host cells. Incorporation of these receptors into a biomaterial may cause the immune system to recognize the material as being part of the host and thus inhibit the immune response [105]. Indeed, Kim et al. have shown that coating polystyrene microbeads with CD200 significantly reduces macrophage activation and the consequent inflammatory response following subcutaneous implantation in a mouse [105]. Kim et al. also suggested that the synthesis of small molecules which possess the essential binding domains of CD200 might result in a product that can be used for coating medical devices to reduce the FBR [105].

24.5.4 Use of angiogenic agents

One of the main issues with the FBR is that the formation of a fibrous capsule around the biomaterial can prevent integration of the biomaterial with the host tissue and can prevent transfer of nutrients into the biomaterial. Addition of angiogenic agents to the biomaterial at implantation can support the formation of a blood vessel network around the biomaterial thus allowing transport of nutrients into the material and increasing the chance of integration with the host tissue [106–108]. Recently the concept of inflammatory vascularization has been established, harnessing the immune response to induce neovascularization of biomaterials [99]. The beneficial effects of this could be twofold, improving vascularization of biomaterials and facilitating host integration [99].

24.5.5 Inhibition of TGF- β /use of corticosteroids

It has been proposed that using a TGF- β neutralizing antibody may block the myofibroblast response to TGF- β , reducing procollagen deposition and thus fibrous capsule formation. However, continuous delivery of a TGF- β neutralizing antibody using a subcutaneous pump for delivery in a mini pig did not reduce fibrous capsule formation around an implanted biosensor [4]. However, inhibition of TGF- β using other mechanisms, for example, corticosteroids, might yield more positive results. Sustained release of dexamethasone (a corticosteroid) from a silicone gel adjacent to a glucose sensor has been trialed in a canine model to assess the effect of corticosteroid administration on the FBR. While there was no significant difference between groups treated with corticosteroids and the untreated control group in terms of length of device function, there was a trend toward increased duration of device function in the corticosteroid-treated group [4,106]. Adjustment of the corticosteroid dose and release profile may enhance the effectiveness of corticosteroids in inhibiting the FBR, however, the significant systemic side-effects of corticosteroids should also be considered when deciding to include them in a medical device.

24.5.6 Mechanical actuation

Actuation of a biomedical implant has the potential to disrupt local fluid flow and alter the attachment of cells and progression of the FBR. Levering et al. reported that increasing the strain rate applied to a urinary catheter reduced bacterial biofilm attachment [109]. Additionally, Cezar et al. have shown that actuation of ferrogels can reduce fibrotic capsule thickness and the number of inflammatory cells in the area around the implant site in a rodent model [110]. Our group have developed a soft robotic actuatable device known as the Dynamic Soft Reservoir (DSR). This device actively modulates the biomechanics of the biotic-abiotic interface by altering strain, fluid flow, and cellular activity in the peri-implant tissue. After 14 days of subcutaneous implantation in rodents, we found a significant reduction in fibrous capsule thickness in the actuated DSR compared with non-actuated controls, whereas the collagen density and orientation were not changed. We also found a significant reduction in myofibroblasts in the actuated group and propose that actuation-mediated strain reduces differentiation and proliferation of myofibroblasts and therefore extracellular matrix production. We found an increase in the number of CD31 + neo-vessels, and a decrease in radial diffusion distance between CD31 + vessels in the actuation group compared with non-actuated controls. Finally, we demonstrate that this reduction in FBR could cause enhanced pharmacokinetics in a porous DSR incorporating a drug delivery component, where we show an improvement in the amount and the area of diffusion at days 8 and 14. This device may act as a versatile tool to further understand, and ultimately to ameliorate, the host response to implantable biomaterials. Of note, in this study the devices were explanted and stained with phosphomolybdic acid (PMA) to enable visualization of collagen dense tissues. Images were acquired using Scanco Medical MicroCT100 and Mimics (Materialise) software was used for segmentation of the device and the fibrotic capsule present. This approach allowed us to generate volumetric reconstructions of the fibrotic capsule, and a thickness analysis was performed to identify the mean fibrous capsule thickness [78].

24.5.7 Monitoring the foreign body response

The effect of fibrous encapsulation on insulin pumps can be measured using blood glucose as a surrogate measure of device function [32]. In intraneural interfaces electrode impedance can be measured by assessing the extent of the FBR [85]. Preclinically Anderson et al. have used bioluminescence imaging to monitor the activity of reactive oxygen species (ROS) in response to implanted poly(styrene) and alginate and found a correlation with ROS activity determined on imaging and the FBR [111]. However, outside of these, no techniques for the real-time measurement of the FBR have reached the clinic [112]. This means that the first sign of implant failure due to fibrous encapsulation may be patient-reported symptoms or a worsening in the patient's clinical condition. This is undesirable and underlines the requirement for better management and monitoring strategies for the FBR.

24.6 Potential uses of the foreign body response

While the vast majority of the scientific literature in this area is focused on reducing the FBR to biomaterials, some interest exists in the potential to elicit a useful therapeutic

response from the FBR. We have previously discussed our study where we sought to take advantage of the host response to mechanically couple an epicardially placed soft robotic sleeve to the myocardium [113]. Another area of note is the area of tissue-engineered blood vessels. The basic concept involves implanting a biomaterial, which prompts the FBR to occur around the biomaterial with eventual breakdown of the original biomaterial leaving a space in the center of the FBR fibrous capsule which subsequently becomes the lumen of a blood vessel [114].

Furthermore, some degree of FBR, in the cardiac context may be useful in increasing blood supply to the damaged tissue and increasing overall cell number in the affected ischemic area [115]. The FBR might also assist in integrating materials with the host tissue. As mentioned earlier, when comparing tissue integration of silicone or medical mesh, the porous nature of the mesh facilitated tissue in-growth thus improving tissue integration compared to the silicone implant [20]. On the basis of this study, we concluded that by altering the surface chemistry of the mesh and the pore size, the mesh could selectively bind to and support heart tissue that has been damaged. Robotic sleeves which assist ventricular function may, in the future be attached to the heart in this manner, reducing the need for sutures and adhesives, ensuring site specific mechanical support, and using the natural FBR to our advantage [20,116,117].

24.7 Conclusion

Biocompatibility testing of biomaterials is a regulatory requirement. Most biocompatibility testing *in vitro* is focused on measuring cell viability following exposure to or encapsulation in a biomaterial. *In vivo* studies often examine the immune response to implanted biomaterials. Protein adsorption onto the biomaterial surface initiates the FBR with subsequent macrophage recruitment and development of a fibrous capsule around the implant. This fibrous capsule can affect diffusion around the implant, integration with native tissue, and can cause patient morbidity and implant failure. Numerous biomaterials have been utilized for cardiac applications and all cause some degree of FBR. The FBR should be considered when designing biomaterial implants and should be investigated early in *in vivo* experiments. If the FBR is problematic for a particular biomaterial implant then strategies such as altering the biomechanics of the device interface, coating the material or incorporation of corticosteroids may be considered to mitigate the FBR. In the future the FBR could be harnessed to assist in vascularization of biomaterials, creation of vascular grafts, and attachment of biomaterials to the surface of the heart.

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Vascular responses to biomaterials

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25.1 Introduction

Developing new therapeutic approaches based on designing novel biomaterials are nowadays paramount for treating vascular diseases. Biomaterials' terminology refers to "any nondrug material suitable for inclusion in the living structure of body, which performs, augments, or replaces a natural function of organs and works in intimate contact with living tissue" [1]. Biomaterials' origin is far back in prehistory, where a stone spearpoint was uncovered in the hipbone remains of a human body [2]. In the 19th century, the first arterial allografts (foreign tissue of the same species) was pioneered and used in human vascular reconstructive surgery [3]. Since blood vessel replacement and artificial heart valves were the focus of many clinical trials in the early 1950s to 1960s, a huge progress was made not only in the therapeutic approach, but also in understanding the mechanisms of interaction between the biomaterials and body components. Despite the positive experimental feedback, the biomaterial-driven regeneration in the complex hemodynamic conditions still have key questions remaining to be answered:

- What is the suitable biomaterial to reduce thrombogenicity, inflammatory reaction and to sustain healing, physiological architecture, and mechanical stability of the tissue?
- Does the deterioration or degradation of the biomaterial affect the healing processes or tissue integrity?

- What are the tools to predict the healing pattern in the presence of certain biomaterials?
- Are all these processes patient-dependent, and therefore personalized therapies being of interest?

The rational design of vascular biomaterials nowadays focuses on the various materials with customized properties. Biomaterial properties need to be tailored to specific applications (internal vs external devices) including the implant duration (temporary vs permanent). Once implanted, the biomaterials can induce a cascade of biological processes which can be devastating [4,5]. Techniques like biofunctionalization or coating, to improve the properties of the biomaterials to sustain specific biological functions and to improve tissue regeneration are still experimental, however they proved to be efficient (e.g., in case of drug-eluting stents). This chapter will focus on the response of vascular tissue to biomaterials and the current trend used to reduce the consequences of this response at the interface with biomaterials.

25.2 Biomaterials in vascular diseases

25.2.1 Biocompatibility

Technical functionality and biological requirements of vascular implants meet the needs for a particular vascular application. On one hand, for vascular implants, biomaterials need to be biostable, wear resistant, and do not deplete in time. On the other hand, the biocompatibility is a compulsory requirement for biomedical implants. In this context, biomaterials must be accepted by the body, should not irritate the surrounding tissue, should not induce any inflammatory or allergic response, should not be toxic or carcinogenic, and should support the mechanical and biological function of the tissue. The compatibility characteristics of vascular biomedical devices include appropriate density, and need to have adequate mechanical properties such as strength, stiffness, and fatigue properties. An appropriate engineering design, long-term storage, and sterilizability are to be included as important parameters for finished-products. In the case of vascular implants, the first aspect to be mentioned is the inherent bifunctionality of the material whereas the biocompatibility one presents an immense interest for companies to develop intelligent technologies from smart materials and receive market approval. Despite of high regulatory compliance needed for market approval and for clinical investigations, biocompatibility tests are a complex task including the physical properties and chemical nature of the implant. Biomaterials' properties are assessed systematically by *in vitro* tests and follow the global normative approach for biocompatibility evaluation. This is done prior to the material reaching the market and being used in the clinics [6]. Synthetic (co-)polymers present increased challenges in the vascular applications due to their lack of biocompatibility, which often triggers inflammatory reactions and fibrous encapsulation. The risk management plan including the biocompatibility plan follows successive evaluation steps, which were systematically reviewed by Bernard et al. [7]: (i) documentation of the complete description of the device, (ii) chemical components of the material, (iii) contact tissue and duration (iv), and population of patients.

The hemocompatibility test is a direct *in vivo* examination of the effect of the implanted device on blood and/or blood components. This test begins in the initial phase of the implantation (endovascular stents, heart valves, etc.) and it examines the thrombus formation, coagulation, platelets, hematology, and immunology (complement and leukocytes). When animal models are employed, species difference between animals and humans needs to be considered, since it may diminish the predictability power of the hemocompatibility test.

Nevertheless, vascular implant-based biomaterials may produce unknown adverse effects lasting for various periods of time after implantation. Toxicity tests estimate the potential harmful effects of the intended substance composing the implants and are used in research and development phases, preclinical studies, and in the quality assurance of the raw material and end-product. Mutagenic tests should test the possibility of the materials to induce changes in local genetic material, while carcinogenetic tests estimate the potential of tumors development induced by the contact of the tissue with the medical device after its implantation or with its degradable components in the case of biodegradable polymers.

25.2.2 Metals and alloys

There are a lot of biomaterials used in clinical applications: biological materials (such as porcine or bovine valves), metals and alloys (such as steel, titanium, magnesium), or polymers (polyesters, polyurethane, polyamides, etc.). In the biomedical industry, implants were initially engineered from pure metals. The bare metal devices displayed poor anticorrosion resistance and poor mechanical strength. Later, these metals were replaced by materials with biochemical inert surfaces. Bioinert materials such as titanium, stainless steel, and cobalt are required for load-bearing functions such as heart valves, stents, and stent-graft combinations. Among these biomaterials, titanium is the choice over, for example, conventional stainless steel or cobalt–chromium alloys. Titanium presents resistance to corrosion and long-term stability while preventing the local or systematic toxicity long term. Their cardiovascular application is in the form of heart valves, pacemaker cans, etc. Preshaping and finishing implants of medical grade titanium-based alloys is a multistep process with employing high manufacturing costs. Mechanically, these inert biomaterials have excellent high strength, fracture toughness, and long-lasting stability under the load-bearing conditions (young modulus: 100–112 GPa, ultimate tensile strength: 785–1050 MPa, yield of stress: 240–950 MPa) [8,9]. Significant proportions of patients receive bare metal devices, mainly based on the antiplatelets therapy duration. A 1-month antiplatelets therapy is sufficient for bare metal stents whereas, for example, drug eluting stents the therapy lasts for 6–12 months [10]. Although iron-based alloys (stainless steel) showed superior mechanical performance, their thick struts resulted in reduced implant flexibility. This characteristic next to a high incidence of acute and subacute stent thrombosis and thereby high doses of anticoagulant drugs created major obstacles for further clinical implementation. Out of bare metal stents existing on the market, metal alloys and stainless-steel alloys are the most frequently used in the cardiovascular context. Alloys based on nickel–chromium and cobalt–chromium have been developed to improve the fabrication characteristics of stainless-steel stents while providing clinical evidence. For example, patients treated with thin struts stents

(<100 μm thickness) presented low neointimal proliferation, reduced inflammatory response at the implantation site, and potentially reduced restenosis [11]. The current stent technology is based on metals with anticorrosion properties. Currently, a huge interest is being addressed regarding biodegradable materials as an alternative for corrosion-resistant metals. Biodegradable materials should avoid late in-stent restenosis, prolonged antiplatelet therapy, mechanical behavior mismatch between adjacent stented and nonstented locations, and an inadaptability to growth in young patients. The candidate material for such applications should provide ideal mechanical performance similar to that of 316 L stainless steel. Magnesium and its alloys, as biodegradable metallic materials, have been the most studied and thereby reached trials in humans. In the 18th century, metallic biodegradable magnesium-based alloy implants showed in vivo a slow degradable profile until complete degradation. Its degradation period proved to be wire size dependent [12]. As the biomaterials undergo progression, iron-based stents have reached the validation phase in animals although it degrades slow for stent application due to a passive layer formation which hinders further oxidation process [13]. Zinc is recently proposed as a biodegradable material in stent applications [14] due to its mechanical performance (yield strength >200 MPa, tensile strength >300 MPa, elongation to failure >15%–18%). Apart from its biomechanical performance, zinc might be preferred over magnesium-alloys due to its machinability characteristics matching classical fabrication procedures (casting and hot rolling). Moreover, in vivo, pure zinc and its corrosion products have had an antiproliferative effect on smooth muscle cells, which did not induce any local necrosis or present other local toxicity [15]. A few elements such as calcium, aluminum, zinc, strontium, and zirconium are employed for reinforcing the mechanical strength of magnesium-based stents, among which magnesium-zinc alloy was validated in the rabbit model as a biodegradable and biocompatible stent and has further been tried in humans [16].

25.2.3 Polymer-based implants

The progress in polymer science for over two decades, has created continuously new opportunities for producing new generations of sophisticated and ingenious biomaterials. Understanding the structure–property–design relationship is paramount for the success of the medical implant performance, with positive consequences on the optimization of clinical treatments and development of more effective and safe cures for a higher quality of human life. As such, the material composition, processing, and the design changes are systematically in accordance to the clinical feedback. Intravascular catheters and mechanical heart valves require unique functional characteristics of employed polymers. The polymer performance is of a wide range which depends on the structural properties of the backbone, their molecular weight, entanglement density, degree of crystallinity, and of crosslinking. The interplay between these properties leads polymers to have a time-dependent mechanical behavior. The required physical properties polymers are: low stiffness, creep resistance, low mechanical strength, resistance to chemicals but easy of shaping. Typically, the elastic modulus and yield strength of polymers increase at increased strain rates while increased load rates lead to polymer failure. Time-dependent mechanical properties of polymers render the in vivo applicability of them, particularly under load-bearing stress devices or even exposed to

the blood flow pressure [17]. But, not only are the advanced high performance of polymers an essential criterion for vascular applications, but their clinical response is an essential criterion for further applications. If they are synthetic or natural, polymer-based materials need to be biocompatible, nonthrombogenic, nonmutagenic, nontoxic.

As synthetic material, high density polyethylene and polypropylene are inert, undegradable, hydrophobic, and very stable, being well tolerated by the human body. Due to their high flex life, they make excellent environments for stress cracking resistance and thereby suited to vascular graft applications. Polyetheneterephthalate has a high melting point, high tensile strength, and great flexibility which make it suitable for molding and therefore used under the trade name "Dacron" in vascular graft, grafts, urological catheters, and for artificial heart valves. Due to its hydrophobicity polyetheneterephthalate is resistant to hydrolysis, microorganisms, fungi, and it is a biocompatible material. Polyetheneterephthalate fibers can easily be molded into mesh-like porous grafts, which can facilitate cell integration into the graft when anastomosed into a host artery. Polytetrafluoroethylene is a fluorocarbon thermoplastic polymer with the brand name "Teflon." Polytetrafluoroethylene is a linear, highly crystalline (90% crystallized molecules), and stiff material with a high melting point, which makes it easy to mold. The very low surface tension, outstanding stretching forces imposed by arterial blood pressure, and inner friction define its applicability for engineering vascular catheters [18]. Plasticized polyvinylcarbonates were one of the first polymers used for catheters, but it showed side effects due to the presence of plasticizers. Nowadays, thermoplastic polyurethanes and its derivatives (polyester-, polyether-based components) as free-plasticizers materials replaced the biomedical application of polyvinylcarbonates. Silicon is a soft material (softer than polyurethane) and thereby can be used as catheters for long term (months) with minimal injury effects. Due to the combination of optical and mechanical properties such as: clearness, toughness, heat resistance, dimensional stability over a wide temperature range, polycarbonates can be an easy mold process. The hydrophobic character, sterilization ability, and biocompatibility make polycarbonates available for medical devices including components of heart-lung bypass machines. For in vivo applications, it is critical to prevent the formation of biofilms on medical devices, since it triggers degradation processes of the material and may cause infections in the human body. In this context, although organic / inorganic biocide additives (silver, quaternary ammonium salts, triclosan, chitosan) have a limited thermal stability compared to polycarbonates (300°C–320°C), they can show efficient antimicrobial/ antibacterial properties [19].

Thermoplastics polyurethane-based shape memory are smart polymer materials. Due to their characteristics (lightweight, high strain/shape recovery ability, easy to process for shape restoration when implanted in the human body) they are proven to be attractive for medical applications including endovascular treatment of aneurysms, miniaturized medical devices which are easily inserted through small catheters and reshape once they reach a specific place. Particularly, they were found to be biocompatible, nontoxic, and nonmutagenic in the human body. Shape memory polymers have large deformation strain, low density, and lower cost than those of shape memory alloys. Stimuli responsive shape memory polymers to magnetic [20], light [21], and electricity [22] fields have been developed in the past years. As such, their recovery behavior includes two-way [23] or triple shape [24] memory in which noninterfering microscopic deformation [deformation vary within the characteristic

distance of the glass transition temperature change: $\delta = \Delta T_g / (dT_g/dx)$] is essential for characterizing the position-dependent shape memory characteristics of the smart material. Modified catheter surfaces with hydrophilic poly(ethylene glycol)-based hydrogel [25] or poly(ethylene oxide) [26] improved their antifouling properties.

Natural polymers (biopolymers) occur either naturally (produced by living organisms) or are made out of raw materials existing in nature. Materials produced by living organisms include proteins such as: collagen, silk, fibrinogen whereas manmade biopolymers include polymer families of linear aliphatic polyesters, polyphosphate esters, polyamides, and polysaccharides. Their biomedical attention is drowning by their ability to repair, regenerate, and replace organs and tissues. Their implantation availability depends on the arrangement and participation of fillers and plasticizers [27]. The catheter's surface gets lubricant to improve the medical maneuver. Polyamide block copolymers are materials used as the outer layer of percutaneous transluminal angioplasty, balloon and stent delivery devices since they combine the polyamide strength with polyurethane's flexibility [17].

25.2.4 Biological materials

Synthetic materials have been widely used in cardiovascular applications. Looking for a perfect synthetic vascular implant, it is important to evaluate whether we are looking too far ahead or we are missing the tremendous potential resources of biological materials required for creating such devices. Moreover, synthetic materials have demonstrated limited biocompatibility and they trigger inflammatory responses. To overcome these limitations, biological scaffolds such as porcine or bovine valves, are proposed as an alternative to the synthetic ones, though this is not to say that traditional vascular devices are not to be used. Considering the required characteristics for implantable devices, the preferable replacement for heart valves and blood vessels is an adaptive, living graft such as allografts and xenografts. These biological materials are living materials that are decellularized allografts made up of extracellular matrix (collagen, fibronectin, laminin, and elastin). However, they still induce a host-immune response and are biodegradable in time (assessment of the leaching components in adjacent tissue), thereby presenting limited applicability as biomedical implants. In contrast to xenografts, allografts are tissues transplanted between nonidentical individuals of the same species, therefore these tissues are of different origins and they undergo specific processing for reducing the rejection of the host. However, using minimally invasive techniques, it is easily shown in sheep that the decellularized de novo engineered valves were extensively recellularized with host cells upon their implantation [27]. Therefore, even still under investigation, the biological materials have a great advantage and are preferred before any other biomaterial for vascular implants.

25.3 Vascular response to biomaterials

In the last few decades, due to the rapid technological progress, the therapeutic strategies to treat cardiovascular diseases expanded significantly. This progress is not due to the development of new drugs, but to the development of biomaterial's field and its

applications. The new implanted valves or stents have higher physiological acceptance, less complication rate, and easier application, therefore they are accepted currently as the gold standard therapy for most of cardiovascular diseases. However, there is still a persistence in inducing adverse biological effects in some patients, mostly of them so severe, or even lethal. Since we do not understand the mechanisms of such adverse effects and how to prevent them, there is a high demand on finding methods to improve and to minimize this as much as possible. Here we summarize the possible effects and current methods used to prevent or cure them (Fig. 25.1).

Cardiovascular health largely depends on the regular work of the intricate and delicate physiological system, and the implantation of an external medical device will break the biological balance and trigger a series of complications. Therefore, an understanding of the biology of the vascular wall and its repair after injury is crucial for the development and use of biomaterials.

The most feared and still unresolved problem of implants that remains is blood clotting. If there is a valve or stent coming into direct contact with the blood it will induce inevitable thrombosis and coagulation cascade. The risk of thrombosis and clotting will

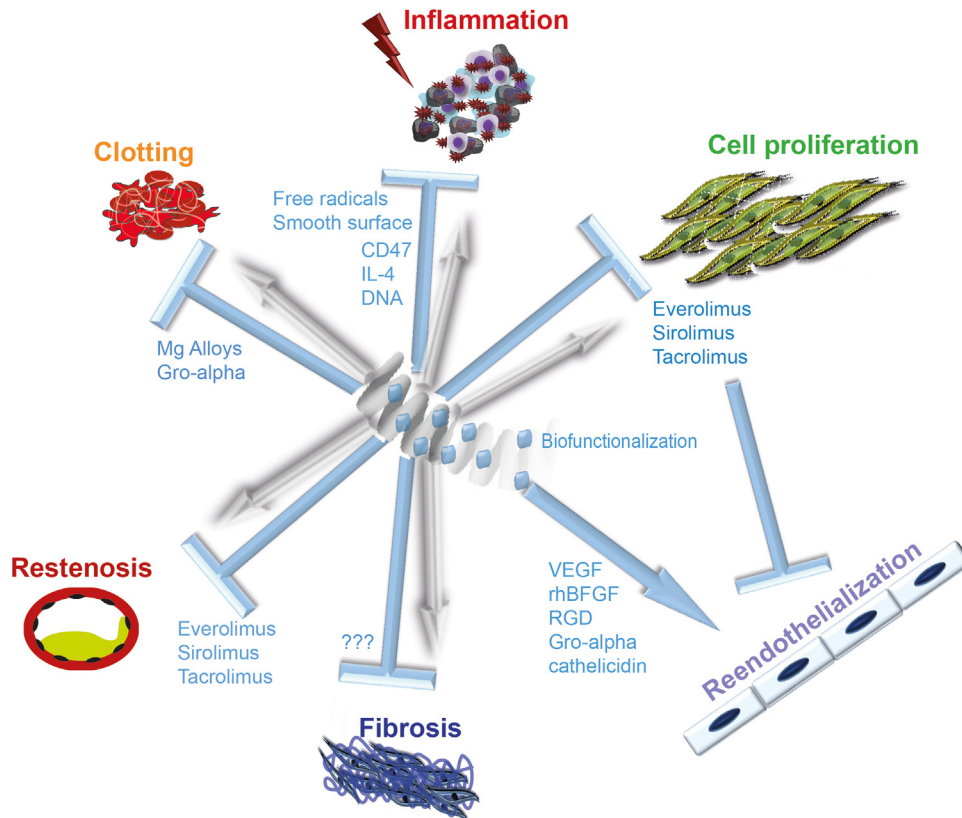


FIGURE 25.1 Vascular response to biomaterials.

persist as long as there are implants present, therefore, the patients need special treatment and are predisposed to complications all of their life. Further, vascular injury is the main consequence of all interventional coronary procedures that induces a cascade of cellular and molecular events which result in acute disruption of the endothelial cells [28], pericytes [29], and smooth muscle cells [30] of the arterial wall. This massive vascular injury will induce a restenosis of the vessel wall, with massive proliferation of the smooth muscle cells and sometimes with reclusion of the vessels. We will also try to summarize our current understanding of the inflammatory and fibrous encapsulation of biomaterials' response in the vascular wall.

25.3.1 Biomaterials and clotting

The most rapid and the most feared complication of the implants is represented by thrombosis and clotting with complete occlusion of the vessel. Rarely, despite sufficient antithrombotic and anticoagulation therapy, some patients still develop thrombosis, and suffer severe complications, such as myocardial infarction or valve dysfunctionality, with fatal consequences. Thrombosis and blood coagulation involve a series of proteolytic reactions resulting in the formation of fibrin clot upon the action of thrombin on fibrinogen. Albumin and fibrinogen are the most abundant proteins in plasma. In considering surface-induced thrombosis, fibrinogen is generally considered to be the central protein in the process of biomaterials-induced thrombosis [31]. The interaction between endothelium and circulating blood components such as fibronectin [32] and von Willebrand factor has been shown to be capable of mediating platelets adhesion to materials.

Factor XII (FXII) is a protease that is mainly produced in the liver and circulates in plasma as a single chain zymogen. Single chain FXII zymogen undergoes activation following contact with negatively charged surfaces of biomaterials. Binding of these anionic surfaces induces a conformational change of the zymogen leading to limited proteolytic activity. FXII is converted into the two-chain activate form, and FXIIa initiates the intrinsic blood coagulation via FXI [33]. FXIIa converts prekallikrein (PPK) into kallikrein and with high molecular weight kininogen as a cofactor activates FXI to FXIa. Factor XIa activates FIX to FIXa. Following cascades of reactions involving among others, the intrinsic complex prothrombin is cleaved to thrombin [33].

Until now there have been no efficient therapies against thrombosis and coagulation activation. Biofunctionalization experiments are very reserved, since a failure can be fatal. Therefore, the therapies using life-long systemic administration of antiplatelets and anticoagulation drugs continue to be the standard procedure in this case. This increases the risk of other complications such as bleeding and impaired surgical interventions.

25.3.2 Biomaterials and acute inflammation

Beside thrombosis and clotting, biomaterials induce a foreign body reaction, an acute inflammatory response caused by the presence of nonnative materials. Foreign materials induce macrophages adhesion and cytokines activation. Most biomaterials come in contact with whole blood when blood is present during the implantation procedure [34,35].

The interaction of blood and biomaterials triggers a complex series of events including protein adsorption, inflammation by ligating innate immune receptors on antigen presenting cells (APCs), platelet adhesion and activation, coagulation, and thrombosis [36–42].

Implanted biomaterials induce a series of events, starting almost immediately to form proteins adsorbed on the biomaterial surface, followed by neutrophils and macrophages attracted to the area in order to provoke an inflammatory reaction eventually leading to fibrotic response. There are many ways in which macrophages might detect biomaterials. It has been shown that macrophages are the primary cells at the inflammatory phase following implantation [43]. Materials like multifilament silk sutures elicited inflammation and were associated with a high number of macrophages and edema. Macrophages' behavior is affected by environmental features [44], including biomaterials properties such as shape and geometry, biochemical surface [38], or composition, porosity, and release of proteins or drugs [45].

Receptors on APCs such as macrophages and dendritic cells will recognize these proteins and trigger phagocytosis [46]. Also, the implantation of biomaterials that are not completely anchored in host tissue will cause enrichment in damage-associated molecular patterns which will adsorb to the biomaterial surface and cause surface-associated innate activation [47].

Since inflammation is a short episode and the biomaterials used for vascular implants are generally inert for our immune system, these antiinflammatory strategies were never considered a priority and therefore, only a few studies demonstrated their potential in reducing complications. However, recently a multifaceted aspect of the inflammatory response continues to be established, not only as inducer of a chain of negative biological processes, but also as a necessity in healing and reparatory mechanisms. In this context, novel potential therapeutic approaches appear and develop in the present with promising results.

25.3.3 Restenosis

Restenosis is an accelerated and severe form of atherosclerosis, when smooth muscle cells massively proliferate as a response to a mechanical injury and deendothelialization [48], inducing rapidly a resistant plaque [49]. While slightly different from the typical atherosclerotic progression, the underlying mechanisms include besides smooth muscle cells proliferation also an inflammatory response, with monocyte adhesion and extravasation and foam cell formation [50], all monitored and controlled by a complex chemokine system [51,52]. However, this process generally ends after regeneration of endothelial layer [53,54]. Some cases of late restenosis are still reported, the assumed underlying mechanisms can be the prolonged mechanical stress induced by the presence of biomaterials in the vascular structures [55–59].

However, the main complications and vascular response are induced by the malposition of the stent and biomaterials, which impaired the physiological movements and function of the organs. Up to 80% of all implanted stents have malapposed struts, and some of them lead to catastrophic events [60]. The research in the context of underexpansion and malapposition is impaired by the limited existing animal models replicating this specific clinical context [60]. Chronic stent enlargement resulted in greater lumen area after

implantation, offering larger diameter of the femoral artery, despite no differences in neointima formation [61].

Biodegradable polymers offer a solution to these concerns. They can significantly reduce revascularizations and late lumen loss, but it seems not to affect mortality or late stent thrombosis [62]. However, uncovered stent struts by neointima and evaginations were less frequent in durable polymers at 2 years after stent implantation in durable versus biodegradable polymer stents, suggesting a significant difference in the very late vascular response in both cases [63]. Recently, a biodegradable magnesium alloy showed up to 6-months structural and mechanical integrity and excellent tissue compatibility, which can represent a promising alternative to traditional metal and polymer implants for the clinical vascular stent application [64]. However, this field is now in full expansion, more and more researchers from all over the world concentrate their efforts to find solutions to overcome these serious shortcomings.

25.3.4 Fibrosis

Fibrosis follows inflammation when biomaterials are implanted. Fibrosis is characterized by the abnormal accumulation of extracellular matrix in the interstitium resulting in impaired and excessive tissue repair. A variety of cytokines and growth factors are involved in the pathogenesis of tissue fibrosis. TGF- β 1 is a profibrotic cytokine that stimulates collagen accumulation. TGF- β 1 induces the differentiation of fibroblast into myofibroblasts and increases collagen accumulation [65]. Cells present at later stages postimplantation (> 14 days) of the foreign body response expressed a mixture of genes associated to M1 and M2 macrophages [66]. Kolb et al. investigated the interplay of fibrosis and inflammation using many different biomaterials. Silicone and poly(lactic-co-glycolic acid) (PLGA) have different levels of inflammation [67]. They measured inflammation by analyzing the percentage of macrophages and fibrosis by monitoring the thickness of the fibrotic tissue around the implant at day 8. Silicon showed the highest levels of fibrosis and PLGA at the lowest. However, both groups elicited similar levels of macrophages at day 3. Inflammation is not predictive of fibrosis after biomaterial implantation. In contrast, several studies have identified important roles in fibrosis for M1 and M2 macrophages. In a separate series of studies, alginate hydrogel cylinders and 0.5 mm spheres implanted subcutaneously in nonhuman primates for 2 weeks which resulted in significantly more macrophage deposition and fibrous encapsulation compared to 1.5 mm diameter spheres [68]. The distribution of triazole-containing analogues creates a unique hydrogel surface that inhibits recognition by macrophages and fibrous deposition [69]. The design of biomaterials to specifically modulate macrophage behavior has emerged as a promising strategy in regenerative medicine and tissue engineering.

25.4 Vascular response to biofunctionalization of biomaterials

The main therapeutic approach to reduce the acute or late response to the biomaterial is represented by local modification of the biomaterial surface to impair or to sustain specific

biological processes. In the field of bioengineering, biofunctionalization is the biochemical or physical modification of a material or the surface coming into contact with the body components to increase temporally or permanently the biological compatibility, reducing the immunological and vascular reaction [74]. Currently, there are numerous approaches to target specific mechanisms by biofunctionalization of the implant, such as antiproliferative, antithrombogenic, reendothelialization, antiinflammatory, and antifibrotic processes (Fig. 25.1).

25.4.1 Antiproliferative strategies

Drug-eluting stents are the first biofunctionalized implants that are widely implemented and used in the clinical practice. Local delivery of antiproliferative drugs like sirolimus, everolimus, and tacrolimus prove to significantly reduce neointima formation compared with bare-metal stents [70,71]. Moreover, intrastent thrombosis in the early phase after drug-eluting versus bare-metal stent implantation in patients with ST-segment elevation myocardial infarction seem to be reduced [72]. However, antiproliferative drugs affect also the reendothelialization and healing of the vascular tissue, therefore the patients need prolonged antiplatelets therapy until 6 months after drug-eluting stent implantation compared with bare-metal stent implantation after only 1 month (clinical practice guidelines from European society of cardiology) [73]. Therefore, the current focus is on creating differentiated coating to specifically accelerate the healing and endothelial cell proliferation, while inhibiting thrombosis and smooth muscle cell proliferation [74,75]. Unfortunately, until now there is no successful experimental setting to fulfill these requirements.

25.4.2 Antithrombogenic strategies

Currently only a few experimental surface modifications are studied to inhibit the thrombogenicity of the biomaterials [76]. Plasma electrolytic oxidation coated Mg–RE and Mg–Zn–Ca alloys [76] or Gro-alpha [77] proved to be efficient in experimental settings. However, to translate this approach in a clinical setting imply a high risk, which nobody wants to assume in the current scientific state-of-art of implant's biofunctionalization. Moreover, clinical practice use efficiently systemic dual-antiplatelet or anticoagulation therapy to avoid thrombosis in the patients receiving implants. Therefore, scientists are focusing at the moment on other mechanisms for biofunctionalization, which can have direct clinical applicability and less risks for the patients by nonresponsivity.

However, even if systemic therapy seems to be efficient in preventing thrombosis in patients, it significantly increases the risk of bleeding, making it sometimes difficult if not impossible for the physician to reach an optimum therapeutic balance between both major risks. Therefore, there is an imperative need to develop alternative strategies, to be able to inhibit the thrombosis in the patients receiving implants, without increasing the bleeding risk.

25.4.3 Reendothelialization strategies

While a few studies are focused on reducing thrombogenicity of the implants, numerous studies focus on sustaining and increasing the reendothelialization [75]. Increasing and

sustaining the endothelial cells regeneration after implantation, will reduce significantly the exposition time of the material to the blood components or other cells, thus reducing the vascular response to the biomaterial. Unfortunately, current drug-eluting stents exert undifferentiated their antiproliferative effect, inhibiting not only the smooth muscle cell proliferation, but also the endothelial cell regeneration. Thus, while the healing after implantation of a bare metal stent is approximated by one month, the healing after drug-eluting stent takes place over six months after implantation, increasing significantly the risk of other complications. Enhancing the reendothelialization reduces the contact period between the blood and cellular components beneath the endothelium, and thus reduces the proliferative stimuli and consecutive, the proliferative processes.

Immobilizing vascular endothelial growth factor [78,79], recombinant human basic fibroblast growth factor [21], arginylglycylaspartic acid peptid [80], Gro-alpha [77], and cathelicidin [81] are only a few examples showing in experimental settings that functionalized surfaces of implanted biomaterials which are effective in specific augmenting of the local endothelial activity, enhancing healing, and reducing complications.

25.4.4 Antiinflammatory and antifibrotic strategies

Other molecules are coated to increase the biocompatibility by antiinflammatory properties [82], such as CD47 [54], interleukin 4 [83], or even DNA [84]. Free radical surfaces [85] or changing even the structure of the surface [86] can represent an effective tool to manipulate the immune cell adhesion. By reducing the local inflammation, the fibrotic processes are also reduced. However, presently, there are no proposed specific antifibrotic strategies.

Unfortunately, all these approaches are still in the experimental phase and all underlying mechanisms of their actions and possible side effects are yet to be defined. However, everybody has agreed that there is a necessity in developing this particular field as the only solution to overcome all the biomaterial shortcomings, with special regards to vascular implants.

25.5 Future perspectives

Despite major clinical advances in the vascular field, the absolute burden of cardiovascular diseases is still high, thus leading to increased morbidity and mortality of patients. This leaves clinicians with the major challenge of applying the appropriate therapeutic strategies for treating and curing vascular diseases. The vascular field is in need of delineating improved future therapeutic options with more precise ability to dictate the optimal regenerative approach. Improved therapeutic strategies highly demand pursuing advanced biomedical devices with high performance and stability, which significantly associates with the development of smart chemistry and the progression in material science field. The emerging applications needs simultaneously conduct the elaboration of smart biomaterials that can deliver the required features with minimal or no side effects on the human system.

It is likely that in the near future, based on biomimetic approaching and using advanced nanotechnologies, multiplex bioinspired and biointegrated devices-based smart hybrid biomaterials will constitute the next vascular devices generation.

It is expected that the synergy between emerging nanotechnologies and the science of human extracellular matrix biology will lead to an ever-growing list of valuable vascular biomedical products which maintain, enhance, or restore vascular tissues. The constant influx of new advances of cell-extracellular matrix or encoded species of extracellular matrix-based biomaterials, will comprehensively bridge the gap among a suite of essential scientific fields in the vascular context. Although several pivotal questions may still remain to be answered, particularly those related to, for example, thrombus formation and proper wound healing, emerging nanotechnologies may uncover aspects related to intimate mechanisms of healing patterns of which, for example, biohybrid human cells/cell-extracellular matrix or encoded species of extracellular matrix-derived smart biomaterials will potentially be rewarded as therapeutic strategies for preventing, for example, thrombus formation. The emerging nanotechnology field will continuously evolve for the collaborative development of more sophisticated biomaterials' designs and inspiration in the future.

Implanted biomaterials induce local inflammation, cell proliferation, blood clotting, fibrosis, or restenosis (*gray arrows*). Biofunctionalization of the material's surface is developing currently to overcome these shortcomings, increasing temporally or permanently the biological compatibility and reducing the immunological and vascular reaction (*blue bars*).

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Bone responses to biomaterials

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Abbreviations

3D	three-dimensional
APC	antigen presenting cells
BCP	biphasic calcium phosphate
BTE	bone tissue engineering
CaPs	calcium phosphates
Cthrc1	collagen triple helix repeat containing 1
EC	endothelial cell
ECM	extracellular matrix
FBR	foreign body reaction
GFs	growth factors
HA	hydroxyapatite
HE	hematoxylin–eosin
HUVEC	human vascular endothelial cell
IFN	interferon
IL	interleukin
Mφ	macrophages
MgHA/Col	magnesium-doped hydroxyapatite/collagen I
MMP	matrix metalloproteinase
MNGCs	multinucleated giant cells
NK	natural killer
PDGF	platelet-derived growth factor
ROS	reactive oxygen species
TCP	tricalcium phosphate
TGF-β	transforming growth factor β
VEGF	vascular endothelial growth factor

26.1 Introduction

The bone tissue is endowed with an extraordinary self-healing capacity as compared to other tissues [1]. Nevertheless, the regeneration success is limited in the presence of large, critical-sized defects, such as those caused by high impact trauma, tumor removal, or congenital defects (e.g., congenital cleft lip and palate), which exceed the regenerative capacity of the bone tissue, or in the presence of pathophysiological conditions, such as old age, osteoporosis, type 2 diabetes, neurofibromatosis, avascular necrosis, and congenital pseudoarthrosis, which are predisposed to fragility fractures and delayed union or non-union owing to functionally impaired host cells [2].

In these diverse instances, bone-grafting procedures are required to restore tissue function and to achieve clinical recovery [3]. The first reports of such interventions date back more than a century, and different strategies have been implemented over time. A key issue in the treatment choice is the decision whether to repair (i.e., to replace a part with something physically similar to the original one) or to regenerate (i.e., to manipulate materials in order to generate a biological structure identical to the one lost or damaged) a bone defect. The gold standard approach is autograft, that is, replacement of the diseased bone with a healthy one taken from a different skeletal site in the same subject, since in principle it contains all the components, in terms of calcified matrix, bone-associated proteins and cells, required to support tissue regeneration; alternatively, allograft or xenograft are often considered [4]. However, the overall benefit deriving from these interventions is undermined by a number of issues, including donor site morbidity, risk of infection or immunogenicity and rejection, limited availability of graft material, low mechanical stability, poor osteoconductivity, need for repeated surgery, and the associated huge economic and societal costs [5].

In this framework, bone tissue engineering (BTE) builds upon the understanding of bone structure, mechanics, and tissue formation to overcome the above limitations of bone grafts and to produce three-dimensional (3D) scaffolds mimicking the extracellular matrix (ECM) and providing the mechanical support that aids in the formation of new bone [6]. This scientific field has grown exponentially since the mid-1980s: a variety of materials and their combination with cells and proteins have been tested in order to find the most appropriate strategy to guide the spatially and temporally complex process of bone fracture repair, thereby generating a functional bone graft [7].

Ideally, the optimal bone-substitute material should be biocompatible (i.e., able to support the presence and function of all the cell types involved in the physiological bone healing process), osteoconductive (i.e., able to sustain new bone growth on the graft), osteoinductive (i.e., able to promote osteogenic differentiation), and osteogenic (i.e., able to support *de novo* bone formation through osteoblasts recruitment) [8–11]. Moreover, depending on the specific site and type of bone defect, the ideal biomaterial should be either stable on a long-term to guarantee prolonged reliability at load-bearing site or resorbable in a timeframe compatible with the timing of tissue regeneration [12]. At the same time, the degradation process should not be accompanied by the formation of toxic byproducts possibly impeding the healing mechanisms [13,14]. With respect to mechanical loads, scaffolds are usually designed to reproduce the mechanical properties of human cancellous bone [15]. They

should also display the degree of porosity, in terms of pore size and interconnectivity, which is needed for cell growth and migration, nutrient flow, vascularization, spatial organization, and osseointegration, and has to be balanced with mechanical strength [16,17]. Nanotopography is an important structural requirement, as well, since it influences the osteoinductivity and osteointegration of the scaffold; accordingly, one of the main challenges for BTE is reproducing the complex structural hierarchy of native bone [12].

Different categories of materials have been evaluated: metals, ceramics and polymers, and their composites [18]. An extensive description of the variety of materials falls out of the scope of this chapter. Here we will just underline that each of them present advantages and limitations, also for what pertains to the elicited cellular events, which indicates that we are far from optimal clinical translation of BTE [19]. For example, metals such as titanium, magnesium and stainless steel, are exploited in load-bearing contexts (e.g., joint prostheses, plates, screws), because of their mechanical strength. The incorporation of silver nanoparticles allows lowering the risk of implant-associated infection, while stiffness reduction through increased porosity allows reducing stress shielding and resorption of the surrounding tissue and improving vascularization. However, metals cannot be degraded or integrated in the host tissue and can liberate wear particles causing inflammation and formation of a fibrous tissue which hamper long-term scaffold success, or other corrosion products which can hardly be eliminated from the body [20].

Ceramics, mostly calcium phosphates (CaPs) (e.g., hydroxyapatite, HA, and tri-CaP, TCP) and calcium carbonate (e.g., bioceramics of marine origin) [21] are biocompatible, osteoconductive and osteoinductive materials with high compressive strength. They are versatile and tunable in terms of degradability and porosity, in order to favor vascularization, nutrient delivery, and bone ingrowth. However, they are brittle and not suited for load-bearing applications.

Synthetic (e.g., polyglycolic acid, PLGA; polylactic acid, PLA; polycaprolactone, PCL) and natural (e.g., collagen, chitosan, alginate, hyaluronic acid, silk fibroin) polymers are highly versatile too. Synthetic polymers lack bioactivity, which limits interaction with the host tissue; on the contrary, natural polymers display ECM binding domains that facilitate tissue integration [22].

All these materials have been tested as such or in combination, and after additional modifications specifically designed to release the inflammatory, angiogenic, and osteogenic key factors known to be involved in the different phases of bone repair, according to spatiotemporally controlled kinetics [23]. Despite the variety of bone substitutes, the tissue responses occurring upon implantation are always similar, comprising different steps, namely, hematoma formation around the graft, necrosis of the graft followed by inflammation and formation of a fibrovascular stroma, graft infiltration by blood vessels and osteogenic precursor cells, and finally new bone formation and resorption [24]. These processes involved active participation of immune cells, endothelial cells (ECs), osteoclasts, osteoblasts, osteocytes and their precursors. Of note, biomaterials can direct cell fate [25], and at the same time, cells can shape their environment [26].

The following sections will provide an overview of the cellular responses to biomaterials designed for BTE (Fig. 26.1). This chapter does not claim to be fully comprehensive, which would be an impossible endeavor based on the continuous and rapid further progress of the field of bone substitutes. Rather the intent is to give an idea of the numerous cellular events involved in the path to a functional bone graft and of the challenges in

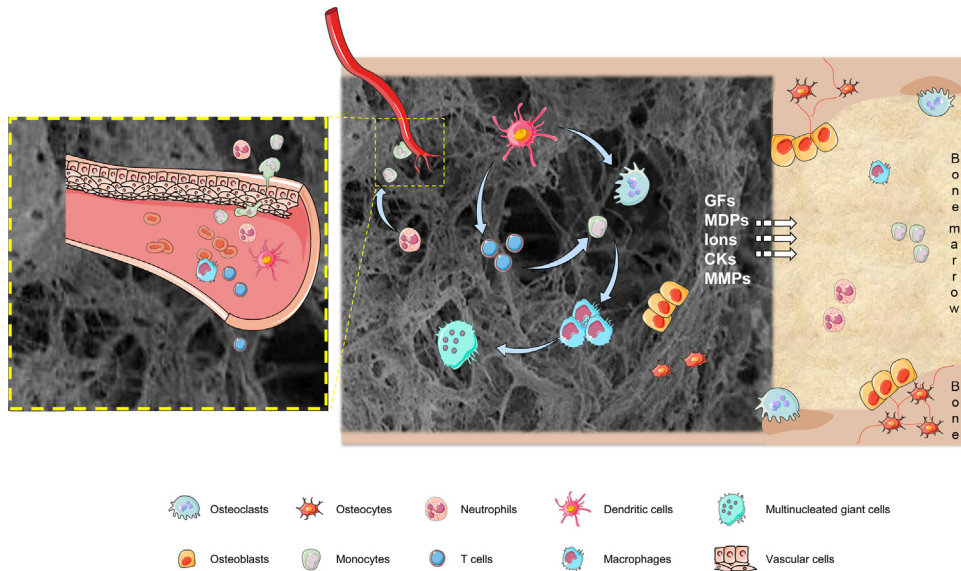


FIGURE 26.1 Simplified representation of resident and recruited cellular players involved in the bone tissue response to biomaterials, depicting the contribution of myeloid cells, lymphocytes, vascular cells, osteoclast, osteoblast and osteocytes. CKs: Cytokines; GFs: growth factors; MDPs: matrix degradation products; MMPs: matrix metalloproteinases.

reproducing the complex microenvironment in which all the cell players can effectively carry out their specific tasks.

26.2 Skeletal cell response to biomaterials

26.2.1 Osteoblasts

Osteoblasts are major players in osseointegration, and graft incorporation depends on the extent of bone formation on the graft itself. The amount of bone apposition on the implant surface is a critical determinant of functional stress transfer, which affects the implant effectiveness [24]. Ideally, bone substitutes should facilitate deposition of the ECM that will replace the scaffold structure over time. In this respect, growth factors (GFs), such as bone morphogenetic proteins, vascular endothelial GF (VEGF), transforming GF β (TGF- β), insulin-like GF 1 and others, play an important role by triggering proliferation and differentiation of osteoprogenitor cells [27,28]. Accordingly, several groups exploited GFs incorporation into the scaffold as a regenerative strategy sometimes attaining variable results [29–32]. Besides, the trophic and regenerative role of GFs constitutes the *rationale* for platelet-rich plasma infusion to improve bone graft integration [33].

The accessibility of ligands displayed by the biomaterial for cell binding is related to scaffold porosity [34]. As the pore size decreases, the surface available for interaction increases, but, under a certain threshold, cells struggled to migrate within the scaffold

itself and pores can be penetrated only by fibrous tissue. Moreover, since osteogenesis *in vivo* depends on vascularization, larger pore size facilitates recruitment and penetration of cells into the graft, along with transport of oxygen and nutrients [35]. The pore size affects also the transfer of mechanical forces to the cells; in this respect, optimal compression and tension on the cell mechanoreceptors has been suggested to be achieved when the biomaterial pores have a diameter ranging from 200 to 400 μm [36]. Overall, in line with bone morphology [37], scaffolds should ideally display a gradient in pore size, in order to provide a balance between cell size, migration, and transport requirements, on one hand, and mechanical properties of the scaffold, on the other [34].

Surface topography influences osteoprogenitor cell fate, osteoblast attachment, and bone formation through engagement of membrane receptors, cytoskeletal modifications, and activation of transcriptional programs [28,38,39]. Rough surfaces have been demonstrated to substantially enhance ECM synthesis and mineralization, even though specific surface preparation techniques may alter cell behavior [36].

Nanostructured surfaces promote cell interaction with specific proteins and thereby enhance bone formation [40]. For example, nanophase ceramics (i.e., materials with grain size $< 100\text{ nm}$) adsorb a higher amount of vitronectin as compared to conventional formulations, and this promotes osteoblast adhesion [41].

Bone ingrowth into porous scaffolds is influenced also by implant stiffness and micromotion between the implant and the host bone [24]. The material stiffness (whether rigid or flexible) determines the local strains occurring within a porous scaffold; for this material property, optimal ranges depend on the specific BTE application [42]. Micromotion is the reversible displacement of the implant caused by the cyclic loading deriving from common daily activities. It induces the formation of a fibrocartilaginous (instead of bony) tissue of varying thickness at the implant–host interface, which can contribute to implant loosening, depending on the type and magnitude of the applied loads, the stiffness and geometry of the implant, and the properties of the surrounding bone [43].

Osteoprogenitor and osteoblast activity can be modulated by the ions incorporated in the biomaterial. Of course, local calcium availability is of outmost importance since it modulates osteogenic precursor recruitment, phenotype, and function, thereby contributing to bone regeneration [44]. Regarding other elements, the elicited effect may vary depending on the chemical entity and amount. For example, carbonate ions can replace either the hydroxyl groups or phosphate ions in HA structure, and, besides the amount of substituted ions, their position is critical with respect to osteoblast growth [45]. Strontium-releasing bioactive glasses and CaP cements increase osteoblast activity and dampen osteoclast resorptive function depending on ion concentration [46]; besides, they have bactericidal properties, therefore they can achieve two goals at the same time, that is, preventing implant-related infections and enhancing bone formation [47].

26.2.2 Osteoclasts

Osteoclasts play an essential role during fracture healing as well as in the remodeling process required for the complete integration of the implanted biomaterial with autologous new bone [1]. Initially, a stable bone-implant surface forms at the implant site;

afterward, the periimplant region has to undergo an intense remodeling, in order to counteract microdamage during functional activity. Lack of physiologic remodeling of an implanted bone substitute that is not intended by design to be permanent, seriously undermines the implantation outcome. Accordingly, Le Nihouannen et al. coated CaP cements with the receptor activator of nuclear factor kappa B ligand in order to enhance osteoclastogenesis and angiogenesis and found improved remodeling of the biomaterial [48]. Thus the authors proposed this kind of strategy to accelerate implant osteointegration after surgery.

The vast majority of studies regarding osteoclast response to biomaterials have been conducted in *in vitro* settings simulating the *in vivo* condition; these experiments served as a basis to predict the clinical performance of biomaterials [49]. On the other hand, conclusions are sometimes discordant among the various reports in literature, likely owing to differences in the adopted experimental conditions [50].

A mainstay is that biomaterial composition strongly influences its degradation, which can occur either through chemical dissolution or through cellular degradation by osteoclasts. The solubility of CaP biomaterials, potentially leading to the continuous increase of these ions in the extracellular microenvironment, may hinder osteoclast resorption [51–53]. Even though the extent of *in vivo* osteoclast remodeling of HA materials has been questioned by some authors [54], in general the dissolution and resorption processes should be balanced, considering the relevance of osteoclast resorptive function in the economy of bone physiology [55]. In fact, factors promoting or impairing osteoclast-osteoblast coupling influence the outcome of the graft [56]. TCP ceramics reportedly have a higher dissolution rate as compared to HA [57], but the resulting local increase in calcium concentration inhibits osteoclast differentiation [58]. On the other hand, HA is more stable, and the combination of different HA/TCP ratios in biphasic ceramics (biphasic CaP, BCP) may optimize osteoclast formation [50,53]. Alternatively, doping apatite with elements such as strontium, iron, silicon, zinc, and magnesium, introduces imperfections in the crystal structure of apatite and results in enhanced dissolution rate and physicochemical performance in biomedical applications [59–62]. At the same time, the presence of metal ions can modulate osteoclast function. For example, strontium hydroxyapatite-containing gelatin scaffolds have been demonstrated to significantly inhibit osteoclastogenesis, while promoting osteoblast viability and activity, as compared to HA-containing scaffolds; therefore a specific application of this kind of biomaterial could be envisaged for the local delivery of strontium in areas with excessive bone resorption [63].

Moreover, the chemical composition of CaP ceramics may influence the crosstalk between osteoclasts and osteoblasts by modulating the expression of positive coupling factors, which direct the differentiation and activation of osteoblasts. In fact, BCP with a low HA content has been found to enhance osteoclast formation and expression of sphingosine-kinase 1 and collagen triple helix repeat containing 1 (Cthrc1) [64]; TCP induced the expression of EphrinB2 and Cthrc1, and octa-CaP promoted C3a expression [53].

Composite materials made of HA and natural polymers offer as an advantage the presence of binding sites for osteoclast receptors [18]; of course, this favors osteoclast adhesion, spreading, and resorption. For example, Taylor and colleagues assessed *in vitro* osteoclast attachment, morphology and resorptive activity on a number of commercial bone

substitutes, differing among them for the presence (or absence) and quality of an organic matrix, HA amount, and surface characteristics [54]. In their experimental set up, osteoclasts could adhere, spread, and actively resorb when cultured on bone-derived materials, likely aided by the presence of noncollagenous proteins in the matrix. On the contrary, they showed reduced resorptive function on synthetic HA and sintered bone, and complete lack on synthetic nonHA materials.

While the relative mineral content does not affect osteoclast adhesion, wettability does: this characteristic of the implant surface determines which proteins present in the micro-environment at the implant site interact with the biomaterial. In turn, the adsorbed proteins form an interface between the biomaterial and the cells, and influence cell adhesion, spreading, and growth [51].

Moreover, the topography of the scaffold surface affects osteoclast development and spreading [65]: rough surfaces have been demonstrated to enhance osteoclast activity by affecting the stability and integrity of the sealing zone [66]; based on this evidence, specific procedures such as sandblasting, acid etching, and grinding are specifically used to increase surface roughness [67,68]. On the contrary, smooth surfaces result in poor osteoclast differentiation [65].

Osteoclastic bone resorption is higher in the presence of small- rather than large-sized HA particles [69] and, accordingly, an addition of small-sized HA particles has been shown to improve the performance of conventional biomaterials [50].

On the other hand, crystallinity, surface bioactivity, and density of the surface seem to have a minor effect on osteoclasts [65].

26.2.3 Osteocytes

Osteocytes are terminally differentiated osteoblasts that become embedded in the bone matrix [70]. Despite their location, they establish a dense network of connections via their cell processes: the more deeply embedded osteocytes connect only to neighboring osteocytes, while recently incorporated (early) osteocytes connect to neighboring osteocytes, to cells lining the bone surface and to cells of the bone marrow compartment [71]. This allows osteocytes to sense mechanical stimuli and tune the opposite activities of osteoclasts and osteoblasts, thereby achieving bone adaptation to mechanical stress through changes in bone mass, architecture, and shape [72]. Mechanical stress importantly influences also the long-term integrity of the graft; in fact, absence of mechanical load triggers bone resorption, potentially impeding graft incorporation.

Nonetheless, in the framework of BTE the osteocytic contribution has long been considered less relevant as compared to that provided by osteoblasts, osteoclasts, macrophages, and ECs [24]. Recently, mostly in vitro but also in vivo studies proposed that osteocytes could be involved in prosthetic aseptic loosening, which is bone loss and prosthetic failure due to bone cell reaction to wear particles [73–77]. In fact, the materials used in orthopedic implants undergo abrasion overtime, and, besides boosting osteoclast activity, the particles formed, independently of their chemical composition (e.g., polyethylene, PE; ultra-high molecular weight PE; TCP; metal ions) [73–77], could stimulate osteocytic osteolysis, as documented by increased size of the osteocyte lacunae in the adjacent

trabecular bone [77]. At the molecular level, several mechanisms have been demonstrated: enhanced expression of catabolic markers, such as matrix metalloproteinase 13 (MMP-13), carbonic anhydrase 2, cathepsin K, and tartrate resistant acid phosphatase [77]; upregulation of proinflammatory cytokines and osteocyte apoptosis [73,75,76]; altered expression of osteocytic markers, sustained increase of intracellular reactive oxygen species (ROS), mitochondrial injury, and Akt inactivation [75].

Overall, these data suggest that osteocytes are indeed active players in bone tissue response to biomaterials. Therefore this aspect would likely deserve further investigation.

26.3 Immune cell response to biomaterials

After *in vivo* implantation, bone-like biomimetic scaffolds interact with the host immune system generating very complex biomaterial–cell interactions, featuring both innate and adaptive immune cells as resident cells of the bone marrow microenvironment or recruited from the periphery to the implantation site [78]; the elicited direct and paracrine effects influence the activities of bone cells during bone regeneration [79]. In general, as a first step in the regenerative process, the inflammatory reaction induces a cascade of biological events that lead to the restoration of bone tissue homeostasis. The characteristics of the immune response are determined by the biomaterial's biophysical properties, such as composition, stiffness, topography, porosity, and geometry [80]; in general, studies with natural ECM seem to indicate reduced immune activation as compared to other materials [81].

After implantation, a temporary blood clot is formed on the biomaterial surface and plasma proteins are promptly adsorbed onto the scaffold leading to inflammatory cell recruitment and activation, mainly cells of the innate immunity including monocytes, macrophages ($M\phi$), polymorphonuclear cells, and dendritic cells (DCs) [24,82,83]. From a molecular point of view, it has been demonstrated that the spontaneous adsorption and thrombin-mediated conversion of fibrinogen on implant surfaces lead to the exposure of specific protein epitopes that interact with the phagocyte integrin Mac-1; this event contributes to phagocyte accumulation on implant surfaces [84]. $M\phi$ in the bone tissue, so-called OsteoMacs, also have a specific function in bone healing and bone regeneration, providing anabolic support to osteoblasts [85,86].

26.3.1 Macrophages

$M\phi$ are among the first cells responding to implanted materials and mediating an inflammatory response. Under specific conditions, inflammation may become chronic and in most of the cases be accompanied by a foreign body reaction (FBR) (Fig. 26.2). The term FBR indicates the host response to the foreign material in the tissue. This process involves mainly cells of the innate, but also of the adaptive immunity; its most apparent feature on tissue histology is the formation of foreign body multinucleated giant cells (MNGCs) (Fig. 26.2A) [87]. First, resident and recruited mononuclear cells react to the implanted biomaterial and differentiate toward $M\phi$ upon cytokine stimulation by other immune cells present in the scaffold/bone microenvironment (Fig. 26.2B). The newly generated $M\phi$

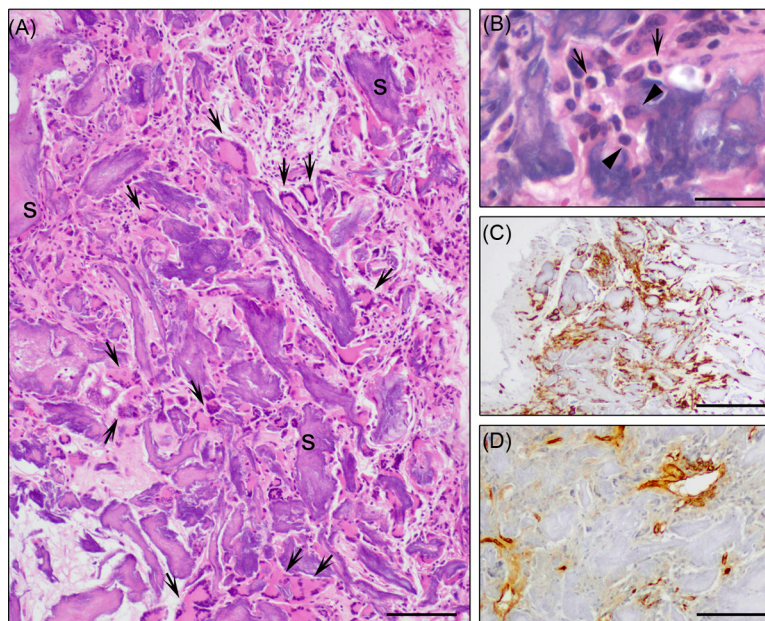


FIGURE 26.2 Representative histological and immunohistochemical images of paraformaldehyde-fixed, paraffin-embedded MgHA/Col scaffold seeded with bone marrow-derived MSCs, after 2 months subcutaneous implantation in immunocompetent mice. (A) HE staining showing the scaffold (s) populated by MNGCs (arrows indicate a few of them), and (B) granulocytes (arrows) and monocytes (arrowheads). Scale bar: 50 μm (A), 25 μm (B). Immunostaining for F4/80 (C), showing infiltrating macrophages (scale bar: 250 μm) and for CD31 (D), indicating vascularization (scale bar: 100 μm) into MSC-MgHA/Col scaffold. HE, Hematoxylin–eosin; MgHA/Col, magnesium-doped hydroxyapatite/collagen I; MNGC, multinucleated giant cell; s, scaffold.

adheres on the biomaterial in order to exert their phagocytic activity as a defense (Fig. 26.2C); in addition, they are the major cell source of MMPs near biomaterials, and the released ECM fragments are instrumental in activating other immune cells [88]. Two different subtypes of M ϕ can be distinguished based on their activation status: the M1 subtype has proinflammatory functions, while M2 exhibits an antiinflammatory or regulatory phenotype. Both the subtypes are involved in tissue regeneration and played a role specifically in the generation of MNGCs [89]. In general, the M1 M ϕ phenotype is involved in pathogen killing due to its proinflammatory and cytotoxic actions and is commonly associated with inflammation clues, while the antiinflammatory M2 M ϕ promotes immunoregulation, tissue repair, and tissue remodeling [90]. Importantly, M1/M2 phenotype ratio can be considered as a parameter to determine the extent of the host FBR upon biomaterial implantation [90,91]. More in detail, within the site of implantation, M1 M ϕ are activated by tumor necrosis factor α and interferon- γ (IFN- γ) secreted by natural killer cells, while lymphocytes induce M2 M ϕ polarization through interleukin-4 (IL-4) production [92]. Of note, the biomaterial ECM can provide different biochemical signals able to enhance tissue regeneration and to promote cell polarization and fusion of adherent M ϕ . For example, bone decellularized ECM–based implants can induce M2 regulatory and antiinflammatory

phenotype, favoring tissue regeneration [93]. Improving the M1 to M2 phenotype switching and the presence of M2 M ϕ at the implant site can be a suitable strategy to enhance tissue regeneration by designing immune-tuning biomaterials [94]. In a recent study, a decellularized bone scaffold was designed to achieve a short release of IFN- γ to promote the M1 M ϕ phenotype, followed by a sustained release of IL-4 to promote the M2 M ϕ phenotype. The sequential M1 and M2 polarization of infiltrating M ϕ resulted in enhanced angiogenesis and healing [95].

Furthermore, biomaterial chemistry can be modulated to harness M ϕ function and polarization. For example, PLA and HA scaffold have been functionalized with magnetic γ -Fe₂O₃ nanoparticles to modulate the function of M ϕ by using magnetic fields driving them to polarize toward an M2 phenotype [96].

If M ϕ are not able themselves to degrade the biomaterial due to high material-to-cell size, they fuse into MNGCs after stimulation by specific cytokines, such as IL-4 and IL-13 produced by the surrounding lymphocytes [97–99]. The degradation of bone substitutes in the proinflammatory environment is accompanied by ROS formation, which plays a role in sustaining inflammation [100].

The generation of MNGCs is strongly influenced also by the biomaterial physicochemical properties; for example, it has been demonstrated that silicon incorporation into polyurethane scaffolds, aimed at increasing the material stability, resulted in the enhanced induction of M ϕ fusion into MNGCs and apoptosis, avoiding M ϕ -dependent degradation of the polymer [101].

The failure of MNGCs in degrading bone substitutes may lead to the expression of platelet-derived GF (PDGF), PDGF-BB, TGF- β , and MMPs by fibroblasts and mesenchymal stromal cells (MSCs), and the subsequent fibrous encapsulation of the biomaterial by myofibroblasts [102]. This can be differently tolerated during bone regeneration depending on the dimensions of the capsule: in particular, a thick one is detrimental to the process [24]. M ϕ does not appear to be important for the generation of the fibrous capsule; on the contrary, they would instruct the surrounding fibroblasts to lower fibroblast-stimulating factors and collagen production [103].

26.3.2 Neutrophils and dendritic cells

In BTE, the first cells engaged at the implant site, characterizing the acute phase of the biomaterial-mediated inflammatory response, are granulocytes, mainly neutrophils (Fig. 26.2B). In this phase, phagocyte recruitment and adhesion to implant is mediated by histamine and favored by fibrinogen, is immediately adsorbed to the surface and replaced over time by other proteins with stronger binding affinity for the material [83]. Neutrophil interaction with the protein agglomerates thus formed and composed of both material-derived and autologous blood-borne macromolecules (e.g., complement components), stimulates neutrophil phagocytic activity; as also occurs in fracture healing [104,105], and the release of granules loaded with several proteases, including caspases which promote apoptosis of adherent cells and their detachment from biomaterials [106]. Moreover, activated neutrophils produce a high amount of ROS [107,108], which may result in damage to the implant and promote M ϕ and monocyte recruitment. Eventually, the tissue can be

infiltrated by lymphocytes, leading to chronic inflammation [83,109]. In addition to their chemotactic effects on inflammatory cells, neutrophils are also able to recruit mesenchymal cells, stimulate angiogenesis, and enhance ECM synthesis [110]. It has been described that neutrophils might have suppressive functions, too, leading to a resolution of inflammation, and, potentially, promoting tissue growth [111]. In the framework of biomaterial response, neutrophils can be influenced from biomaterial composition. In a recent work, polyacrylonitrile/HA/bovine serum albumin (BSA) composite has been used to obtain modified carbon nanofibrous scaffold sheets for BTE application. The *in vivo* study in rabbit demonstrated that the sheets functionalized by HA/BSA were able to recruit a low number of neutrophils in the tissue area surrounding the sheet, as compared to HA-coated sheet or carbon nanofibers alone, rendering the new composite more biocompatible [112].

Furthermore, DCs are able to infiltrate the implanted biomaterial and can also derive from monocytes differentiated at the site of injury or inflammation [113]. DCs directly respond to ECM components or to soluble danger signals released by necrotic cells and adsorbed to the biomaterial, recognizing these ligands by means of Toll-like receptors which downstream activate the MyD88 and NF- κ B pathways [114]. However, a different cell behavior is elicited depending on the biomaterial. For example, DCs adhere strongly to fibrinogen and PLGA, but not to collagen or laminin, and this results in upregulation of multiple integrin dimers [115]. They are poorly activated by alginate and hyaluronic acid, while strongly induced to upregulate costimulatory and major histocompatibility complex (MHC) molecules and to secrete proinflammatory cytokines in the presence of PLGA or chitosan [116,117]. DCs can actively participate in the FBR; in fact, they have phagocytic capacity and can uptake CaP particles and secrete specific inflammatory cytokines influencing the immune response to the implanted biomaterial. For example, when polyvinyl sponges were implanted subcutaneously in rats, DCs increased over time at the site of implantation, and suppressed chronic inflammation and the autoimmune reactions to damaged tissue, suggesting that DCs might be relevant for the resolution of the immune cell response [118]. However, it is not clear whether these cells interact directly with the biomaterial or act via M ϕ and MNGC. Moreover, in the presence of CaP-based scaffold, DCs might also migrate back to the lymph nodes to prime T cells activating adaptive immune reaction [116]. Thus DCs represent the link between the foreign implanted biomaterial and immune cell response, modulating the inflammatory status of the corresponding tissue area and contributing to bone regeneration. DCs have been shown also to respond to biomaterial surface chemistry, hydrophobicity, and topography [117].

Of note, DCs are able to differentiate into bone-resorbing osteoclasts in pathological settings [119,120], specifically upon stimulation by granulocyte-macrophage colony-stimulating factor and IL-4, which are factors present in the surrounding implanted bone tissue environment [119,121]. So, overall DCs might be involved in inducing bone resorption during bone inflammation and regeneration.

26.3.3 T cells

It has been demonstrated that T cells are involved in cytokine production and tissue regeneration [80,122], although the exact function in the context of the immune response

to biomaterials is still poorly addressed. T lymphocytes are recruited to the site of implantation by antigen presenting cells (APC; mostly DCs) or by M1 M ϕ during the inflammatory phase of the FBR [123,124], and several T cell subpopulations can induce M ϕ polarization and MNGC formation [125].

However, the adaptive immune response has been demonstrated to occur mainly when biomaterial implantation is coupled with allogeneic cell transplantation for bone tissue regeneration [126]. In this framework, the MHC class I and II molecules, expressed, for instance, by MSCs, present the antigen to the CD8⁺ and CD4⁺ T lymphocytes [126].

Upon implantation of cellularized constructs, they can be directly recognized by the host immune system. Different factors may influence the degree of T cell response, such as the type of implanted cells, the implantation site, and the immunological status of the host; moreover, the ratio between CD8⁺ and CD4⁺ T cells may vary depending on the presentation modus, either direct or indirect antigen (i.e., by MHC or APC, respectively) [127]. Proinflammatory cytokines produced by CD8⁺ T cells induce the skewing of CD4⁺ T cells toward a Th1 phenotype, and both CD8⁺ T cells and Th1 participate, eventually, in the lysis of donor cells [128]. Conversely, the attracted Th2 cells boost the recruitment of eosinophils that once activated, might be responsible for tissue damage and rejection of the implant [128].

It has been demonstrated that when chitosan is used as biomaterial, activation of adaptive immunity occurs since the biopolymer enhances antigen-specific T helper 1 (Th1) responses in a type I IFN receptor-dependent manner [129]. On the other hand, 3D interconnected porous chitosan–silica (CS/SiO₂) and CS/SiO₂/HA hybrids have been produced for bone defect repair. When implanted intramuscularly, CS/SiO₂ and CS/SiO₂/HA hybrids induced a local and limited monocyte/macrophage infiltration. In particular, adherent DCs were not activated to prime an adaptive immunity response, as demonstrated by the absence of cytotoxic T cells in the implanted tissue [130].

In the context of nonautologous chondrogenically induced MSC employed for BTE, it has been demonstrated that MSC expressed intermediate/low levels of MHC class I, while very low to no expression of MHC class II, allowing them to escape CD4⁺ T cells immunosurveillance [131,132]. Moreover, chondrogenic-induced MSC are able to influence T cell polarization turning Th1 and Th17 cell subset in regulatory T cell phenotype [133,134].

In general, cells belonging to the adaptive immunity mostly mediate host response to allogenic cells, while the innate branch of the immune system is the main player of bone cell response to biomaterial implants.

26.4 Vascular cell response to biomaterials

In general, tissue damage generates a hypoxic environment due to the destruction of the local vascular network. Bone defects exhibit this hypoxic condition, too, and bone regeneration relies on adequate vascularization, providing oxygen, nutrients, osteogenic and osteoclastogenic precursor cells, and eliminating waste products [1].

In fact, scarce blood perfusion results in poor survival of embedded cells owing to the lack of oxygen and nutrients, and accumulation of waste products, on one hand; and insufficient recruitment of endogenous cells expected to contribute to the healing process,

on the other. This can ultimately cause lack of graft integration into the recipient site and graft failure [135]. Graft size and contact area with circulation are critical aspects: large grafts take a long time to be vascularized; in the meantime, necrotic area can form in the inner part.

Sufficient implant vascularization requires a network of large and small vessels expected to develop inside the scaffold (which can be achieved *in vitro* too) (Fig. 26.2D) [136] and anastomose to the host circulation [137].

Autogenous cancellous and corticocancellous bone grafts present a porous architecture that favors vascular invasion, but their use is associated with the drawbacks mentioned in the introduction.

In BTE a wide variety of materials and production methods have been conceived to optimize construct vascularization [138], including biomaterials enriched with GFs or other bioactive molecules [139,140]; scaffolds with larger pore size to facilitate vascular invasion [34]; materials modified with the RGD or the laminin-derived YIGSR peptide increasing EC adhesion, proliferation and migration; bioprinted scaffolds containing a functional vasculature [135]. In this respect, for example, a recent report described the production of a scaffold made of an electrospun polymer constructed into both bone-like trabecular and cortical components with varying porosity and shape, in which the cortical channels were prevascularized by seeding immortalized human microvascular ECs (HMECs) [141]. After 2 weeks, the HMECs were eliminated from the scaffold by means of freeze–thaw cycles, and other cells, namely, MSCs, were seeded. The authors observed that, even though HMECs did not form mature vessels, they nevertheless produced a vascular matrix which functioned as an angiogenic environment, as suggested by the increased presence of VEGF, CD31, and VE-cadherin in the scaffold [141]. Alternatively, ECs *in vitro* culture under dynamic conditions in a perfusion bioreactor have been applied and showed improved cellular density and a number of lumen structures formed by ECs in the inner part of the scaffolds, as compared to static culture; therefore this option might have the potential to generate clinically sized, vascularized bone scaffolds [136].

The angiogenic potential of ECs has been tested in the presence of different types of materials. For example, it has been shown *in vitro* by scanning electron microscopy that human ECs attached to the surface of poly(lactide-*co*-glycolide) microspheres used to develop biomimetic porous resorbable scaffolds, and formed bridges between single microspheres [142]. The cells also formed contacts among them and maintained their phenotypic characteristics independently on pore architecture [142]. In another study, the human umbilical vein endothelial and human pulmonary microvascular EC lines [respectively, human vascular endothelial cell (HUVEC) and HPMEC-ST1.6R] served as a model of macro- and microvasculature on starch and PCL fibers, previously shown to support the viability of bone marrow cells. ECs proliferated and maintained a normal morphology, expression profile, and response to proinflammatory stimuli [143]. Furthermore, the ECM-like structure of the fibers guided EC migration and organization into small vessels resembling capillaries [144]. HUVECs were shown to adhere and spread along silk fibroin fibers, a material used to produce porous scaffolds, and to form microvessel-like structures on fiber nets embedded in a collagen type I gel [145].

Finally, bone and immune cells supported implant bed vascularization through the expression of pro-angiogenic GFs, *in primis* VEGF [146]. Accordingly, strategies for

engineering prevascularized bone grafts by coculture of endothelial and bone-forming cells, have gained interest [147,148].

More recently, the study of the angiogenic response to implantation has been enabled by specific animal models, in particular the cranial window and the femur chamber models in mice and rats, allowing a time-course microscopic view of cellular interactions through observation chambers put close to the implant [149–151]. By means of intravital fluorescence microscopy, microhemodynamic parameters can be repeatedly measured over time to assess the establishment of a functional vasculature prior to histological analysis. Despite their complexity, these models might significantly contribute to identify materials with optimal performance upon implantation.

26.5 Conclusion

Clinical situations requiring bone-grafting procedures or BTE are increasingly common, in part owing to the general population aging and the health-related consequences. The growing medical need has fueled the expansion of the field of bone regenerative medicine, which on the other hand has highlighted the importance of the triad “scaffolds-cells-signals” in tissue engineering [152]. Technological advances have led to a wide understanding of the various hierarchical levels featured by natural bone tissue, which constitutes the starting point to implement BTE strategies. The increasing knowledge of the compositional, structural, and mechanical features influencing cell functions has enabled designing proactive biomaterials (i.e., materials potentially able to elicit specific, timely, and desirable responses from surrounding cells and tissues) necessary for improved implant efficacy. We envisage that a multidisciplinary, holistic approach combining osteointegration, vascular integration, and tuning of the immune response into a single system will enhance the chances of success of BTE products when applied in the clinical practice.

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Tendon and muscle responses to biomaterials

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27.1 Introduction

Tendon and muscle injuries are clinical problems that are increasingly common due to an aging population and an increasing life expectancy. The injuries also common in young and middle-aged individuals involved high-demand physical activities or in situations involving physical strains and risks. These injuries comprise a spectrum, ranging from inconsequential sprains to complete ruptures. Minor sprains and degenerative tendinopathies in general are treated conservatively, such as with immobilization and splintage, oral medications, and various types of injections. For complete tendon and muscle ruptures however, depending on the site of injury, treatment modalities often entail surgical management, ranging from primary suturing with or without tissue grafting and augmentation, to reconstructions with bioengineered constructs and materials. Despite the numerous available treatment modalities with various efficacies, it is rare for current therapies to date to be able to return patients fully to their premorbid function. This is in part due to the poor regenerative capacity of tendons, and to the potential for scarring and wasting of injured muscle. This clinical conundrum has led to the rationale for developing innovative solutions for functional tissue therapies.

Whether utilizing standard suture techniques, tissue grafting or bioengineered tissue repair, and regenerative approaches, the application of biomaterials is a feature in the surgical treatment of tendon and muscle injuries. Biomaterials are defined as any substance, other than a drug, or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ, or function of the body [1], and in the context of tendons and muscles, the relevant materials encompass surgical suture materials, process and engineered substances derived from natural sources, and synthetic materials for reconstructive purposes. As with any foreign material implanted into the body, these biomaterials may

elicit various reactions, both beneficial and deleterious, that are influenced by the material properties as well as by the host tissue and status.

27.1.1 Composition of tendon and muscle tissues

Tendons are dense fibrous sheaths of connective tissue that attach muscles to bone. They play a vital role in force transmission between musculoskeletal tissues. The histological composition, geometrical, and conformational alignment along with orientation of its fibers are of utmost importance to fulfill its function. Tendons are composed mainly of water (~50% of wet weight). They comprise an extracellular matrix (ECM) and a cellular component.

The ECM consists of collagen, proteoglycans, and glycosaminoglycans. They make up the load bearing portion of tendon tissue. Type I collagen makes up 85%–90% of the tendon's dry mass. Type I collagen molecules classically aggregate to form fibrils in a triple helical heteropolymer, the basic nanostructural tendon unit [2]. Bundles of fibrils form fibers, fibers group into fascicles and fascicles bundle within endotenon sheaths to form larger bundles surrounded by the epitenon. These fibers are oriented in a wave-like pattern. Smaller amounts of other collagens are also present, such as Type III, V, XII, and XIV. Type III collagen is naturally found in the endotenon and epitenon. Aging and tissue damage reduce its content, causing a reduction in tendon elasticity.

Tendons have limited cellular content, vascularity, and innervation. The cellular component encompasses a majority of elongated specialized fibroblasts, called tenocytes (~90%–95%), of which the precursor is a tenoblast. In young tendons, tenocytes are abundant and found in close proximity to developing collagen fibers. In mature tendons, these tenocytes become less metabolically active, and they flatten, shorten, and diminish in number. The remaining 5%–10% of cells have tendon stem cells (TSC), chondrocytes, vascular endothelial cells, synovial cells, and smooth muscle cells.

Tendon joins skeletal muscle to bone (Fig. 27.1). Skeletal muscle is a very dynamic tissue, and is abundant constituting about 40%–50% of human body [3]. With the help of tendons, skeletal muscle transmutes chemical energy to mechanical movements of bone, supporting the locomotion, breathing, and postural maintenance of the body. Muscle is a highly organized construct comprising parallelly arranged myofibers, a type of multinucleated muscle cell. The myofibers are held in place by connective tissue which is mainly composed of collagen Type I, collagen type III, and proteoglycans [4]. Unlike tendons, skeletal muscles are physiologically active with good regenerative capabilities, mostly because of the presence of resident multipotent cells which facilitate muscle regeneration in response to tissue damage [3].

27.1.2 Injury and healing of tendon/muscle

Common tendon injury sites are the rotator cuff tendons, hand flexor/extensor tendon, and patellar tendon. These tendon injuries were once thought to be primarily from inflammation but with advances in imaging techniques and histopathological analysis, it is now widely accepted that these injuries are more degenerative in nature. These degenerative

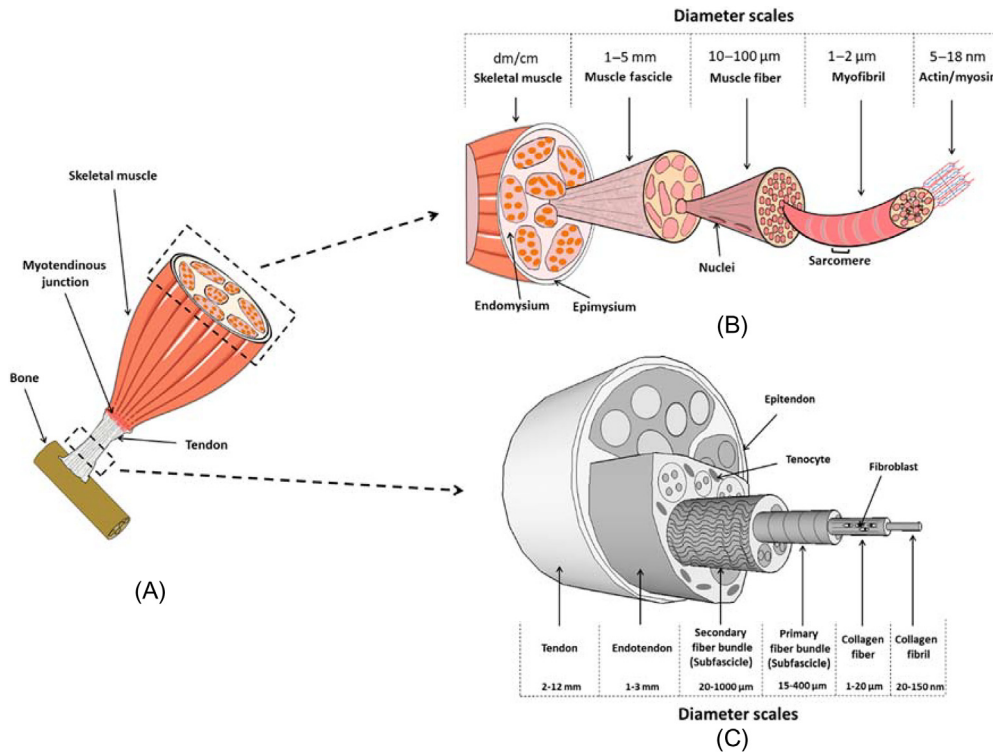


FIGURE 27.1 Schematic representation of a bone–tendon–muscle continuum (A), microarchitecture of muscle (B), and tendon (C) [3]. Source: *Distributed under a Creative Commons Attribution License 4.0.*

tendinopathies are typically characterized by mucoid degeneration on imaging modalities and demonstrate islands of high cellularity, tissue disorganization, and neovascularization on histological analysis [2].

After an acute/chronic repetitive injury to a tendon tissue, healing occurs. The wound healing process occurs in three steps: inflammation, repair, and remodeling. In the inflammatory phase, inflammatory cells migrate to the site of injury and mitigate phagocytosis of unhealthy debris. In the repair phase, cytokines signal for fibroblasts to proliferate, synthesize, and deposit ECM [2]. In the remodeling phase, newly synthesized collagen fibers are aligned longitudinally to achieve their load bearing function. Any interference or alteration to this process, such as surgery and the introduction of biomaterial foreign bodies, can lead to less organized healing and deposition of scar tissue that can result in suboptimal function.

Muscle injuries can be sports-related leading to contusions or strains [3]. Injuries can also be caused by trauma or surgical removal which may lead to volumetric muscle loss. The healing of muscle tissue occurs in three distinct phases, namely, the destruction phase, repair phase, and remodeling phase. In the first phase, the ruptured myofibers contract and the damaged muscle is enveloped by hematoma leading to inflammation. The repair

phase is marked by the clearance of debris by cells of monocyte lineage, which is followed by differentiation of satellite cells to myoblasts. Thereafter, myoblasts undergo proliferation for about 24 hours before they fuse to form myotubes, which develop to become myofibers. The whole process takes about 5–6 days. Lastly, in remodeling phase the newly formed myofibers mature to produce a functional muscle tissue.

27.2 Management of tendon/muscle injuries and responses

27.2.1 Suture

For both primary tendon suture and grafting techniques, sutures remain the mainstay of maintaining initial structural integrity while the healing process progresses. Sutures should have extensive mechanical strength which will help tendons to transmit forces. Various suturing techniques are employed to enhance repair strength. The two main complications of tendon repair are risk of rupture and adhesion formations [5]. To prevent adhesion formations, early mobilization is encouraged, but this may increase the risk of re-rupture. The repair strength can be improved by increasing the number of strands of suture; however, the tension caused due to the suture grasp can lead to cell death, leaving an acellular zone around the suture [6]. This can also lead to sustained inflammation promoting adhesion formations. Detailed investigations of these biomaterials are needed to understand the pathophysiology of ruptures. Polyethylene-based sutures with silicon and polyester coating such as Fiberwire are widely used for musculoskeletal repair [7]. Although the sutures have excellent mechanical properties, the material can induce severe granulomatous reactions with giant cell formation. To improve cellular responses to suture materials, a strategy of commercially available polyester/polyethylene suture coated with collagen type I has been used, and the technology has been shown to improve osteoblast adhesion and differentiation on the suture in in vitro setting [8].

Sutures used for skeletal muscle repair have been assessed for their immune responses and healing of thigh muscle in albino rats. Both resorbable [polydioxanone (PDS) catgut and polygalactin 910] and nonresorbable (nylon) were assessed in the study by Bhargava et al. [9]. Nylon being nonresorbable demonstrated high levels of lymphocytic proinflammatory responses 48 hour postsurgery, whereas bioresorbable sutures, namely, catgut, polygalactin 910, and PDS displayed comparatively lower inflammatory responses. Of all the sutures, PDS had the lowest inflammation. One-week postsurgery collagen-based catgut suture displayed macrophage infiltration. This might be beneficial for wound healing but the suture loses mechanical strength at early stages, hence, it is not desirable for the healing of dynamic muscle tissue. Apart from being nonimmunogenic PDS has very low degradation rate hence the suture retains tensile strength and is absorbed gradually over 180–210 days. In a separate study, tissue reactions to biodegradable suture materials (catgut thread, DemeTECH polyfilament thread, and Surgilactin monofilament thread) were investigated in subcutaneous fat of rats [10]. Distinct stages of initial inflammation, and subsequent tissue healing with development of new vessels and connective were observed for all the sutures. However, complete suture absorption and tissue restitution was only observed in DemeTECH polyfilament thread 12-month postsurgery. Hence the choice of

suture materials affects the tissue healing and host immune responses. Detailed research is needed to understand the cellular mechanisms and pathophysiological implications of host responses to suture materials.

27.2.2 Tissue grafting

Tendon suture techniques form the backbone of standard tendon injury management. For large/complete tendon ruptures, the current gold standard of management is reconstruction of ruptured tendon using grafted tissue [2,11]. The grafted tissue could be autologous, allogeneic, and less likely xenogeneic. Likewise, for the repair of muscle loss of greater than 20% where self-repair is compromised, autologous graft transfer such as muscle flap remains the gold standard treatment regime [3].

The use of autologous grafts has shown tremendous functional results for decades. However, donor side morbidity remains an unresolved concern [12]. Issues related to the use of allografts include significant failure rate of up to six times that of autografts [13], and cost [14]. To circumvent issues related to high cost and donor site morbidity, clinicians had turned to the use of xenografts. Even though short-term functional results were promising, implant failure rate in the medium to long term was significantly higher and possibly attributed to tissue processing, immune rejection, and decellularization [2].

Xenograft mimics the natural tissue architecture and is procured from animals. The supply is not as limited as allografts, though inflammation and foreign body response (FBR) are associated with some xenografts [15]. Some commercially available grafts are Restore, Bio-Blanket, TissueMend, Encuff Patch, Shelhigh No-React Encuff Patch, Graftjacket, and OrtoADATP [16].

In patients treated with porcine small intestine submucosal grafts for rotator cuff injury, early inflammation was observed. Histological examination revealed acutely inflamed granulation tissue filled with neutrophils, but no giant cells, crystals, eosinophils, or pathogens were present [17]. In a separate comparative study, five commercially available grafts, namely, GraftJacket, Restore, CuffPatch, TissueMend, and Permacol, used for orthopedic soft tissue repair were examined for host response in a rodent model [15]. Restore graft and autologous graft (control group) displayed rapid degradation and high cell infiltration. The grafts with delayed degradation profile, namely, CuffPatch, TissueMend, and Permacol presented with chronic inflammation with multinucleated giant cells which are responsible for FBR. GraftJacket and Restore grafts, however, were infiltrated by mononuclear cells. Neutrophils are associated with acute inflammatory response whereas mononuclear cells are linked to subacute and chronic inflammation [18,19]. Normally, mononuclear cells follow neutrophils to reach the site of inflammation. They phagocytose the foreign particles and exit from the site; such response was perceived for autologous graft used as the control [15]. However, cellular activation was different in the commercial grafts. Each commercial graft exhibited distinct cell stimulation profile. The differences in immune cell response might be contributed to the differences in graft source and processing treatment.

To minimize the risk of immune response, the noncollagenous counterparts such as fat deposits, cells, and debris are removed from the graft [20]. Depending on the nature and

source, the graft is chemically cross-linked, laminated, or lyophilized, etc. [21]. The processed graft predominantly comprises collagen type 1, the texture is bioactive which supports the cells infiltration and proliferation. Yet, the host responses, associated risks and benefits of the decellularized grafts have to be thoroughly investigated for clinical use.

27.3 Regenerative strategies for tendon/muscle injuries

Regenerative medicine and tissue engineering strategies have been widely explored to treat tendon/muscle tissue injuries. Low metabolic activity, vascularity, and cellularity weaken the self-healing capabilities of tendon tissue [22]. Hence, the treatments are focused to boost the body's own repair and regenerative mechanisms, by administering cells, growth factors, genes at the site, or by restoring the functions of damaged tissue by using grafts or synthetic scaffolds. Research has been conducted both in *in vivo* and *in vitro* settings to access the benefits of administering biological and biomaterial-based regeneration therapies, aiming to reiterate the natural healing mechanism of tendon tissue [23].

27.3.1 Hydrogel biomaterials for small tissue repair

Stimulation of *in situ* tissue repair and regeneration, by administering viable cells, genes, protein directly at the site of injury, is one of the rapidly developing technologies in tendon tissue engineering. Delivering cells that are capable to produce tissue matrix to a hypocellular collagenous connective tissue such as tendon facilitate tissue formation and remodeling [24]. Various cell types were investigated to stimulate tissue healing. The choice of cells varied from tissue specific tenocytes and TSC to nontissue specific cells such as mesenchymal stem cells (MSCs) and adipose-derived stem cells (ADSC). Primary myoblasts, satellite cells, fibroadipogenic progenitors, and human pluripotent stem cells possess high regenerative capabilities, hence are appropriate cell source for muscle tissue engineering [3]. Short half-life of the injected cells and inadequate research to define the culture conditions for cell expansion and phenotype preservation are some challenges that need to be addressed.

Exogenous genetic materials are delivered to the cells and long-term protein expression can significantly improve the repair site [23]. Therefore multiple injections and administration of higher therapeutic doses for exogenous entities can be avoided. The delivered genes produce proteins by natural mechanisms; hence, they have better biological responses with reduced risk of eliciting adverse immune reactions [25]. In spite of these benefits, short lifespan of nucleic acids in plasma and poor uptake of nucleic acids by host cells are some drawbacks of gene delivery. To facilitate efficient gene delivery, carriers such as liposomes, polymersomes, and adenovirus vectors were explored [23]. By gene delivery, desirable modulation of the gene expression of tenocytes could be achieved in *in vitro* settings [26]. Scaffold-based gene delivery has also been shown to retain the desired protein production at a sustained manner by the targeted cells [27]. Moreover,

gene delivery augmented tendon healing with improved mechanical properties in in vivo animal models [28,29].

Conversely, gene therapy is limited by safety concerns such as risk of immune responses and mutagenicity associated with viral vectors [30]. The three main viral vectors commonly explored are adeno-associated virus, adenovirus, and lentivirus. Each vector has its own responses to the innate and adaptive immunity. Adeno-associated virus has a low and transient response by the innate immunity, but adaptive immune responses depend on the target organ, mode of delivery, and dosing schedule [31]. Adenovirus stimulates a strong innate immune response, while both adenovirus and adeno-associated virus have preexisting neutralizing antibodies that limit cell transduction [32]. Lentiviral vectors, though able to express transgene long term, have an associated risk of carcinogenesis as it inserts into the cell genome [33]. Nonviral vectors are comparatively safer but have poor transfection activity [34]. In mammalian cells, synthetic vectors' capability to deliver the therapeutic nucleic acid pale in comparison to that of viral vectors. Nevertheless, the progression of nanotechnology, as well as new polymers, had allowed to yield nanosized delivery vehicles [34]. Currently, nonviral gene therapies are not approved by US Food and Drug Administration (FDA), but it has already garnered significant interest for further development. Further research is needed to design appropriate carriers for nucleic acids.

The administration of exogenous growth factors and cytokines to stimulate tendon healing and regeneration is an evolving clinical approach [23]. These growth factors aim to signal cells for chemotaxis, proliferation, differentiation, matrix deposition, and finally, healing. The current applications are through local injections, sutures, and scaffolds [35]. Bone morphogenetic protein, platelet-derived growth factor, vascular endothelial growth factor (VEGF), fibroblast growth factor, and transforming growth factor beta (TGF- β) are few proteins identified to improve almost all the sequential phases of tendon healing [23]. Administration of VEGF improves vascularization and boosts early tendon healing whereas TGF- β improved cellularity and biomechanical properties of tendon tissue [36,37]. Although the treatment is advantageous, optimal dosage and the number of administrations are still not defined. The technology is also limited by appropriate mode of delivery and short half-life of these proteins in physiological conditions [23]. Pertinent delivery vehicle is needed for the sequential release of growth factors, which would then help to reduce the treatment dosage and number of injections at the site of injury. Biological approaches are now explored in conjunction with delivery vehicles.

Hydrogels have been widely explored as a delivery system to encapsulate cells, growth factors, and other regenerative morphogens. The hydrogel system is porous with water retention capabilities. The polymeric networks can be fashioned with biomimetic physiochemical cues to mirror the ECM of native tendon tissue. Moreover, the mechanical strength and rate of degradation of the hydrogel can be fine-tuned via a number of cross-linking procedures which can be based on temperature, pH, light, or chemicals [38,39]. Hydrogels can be also used to produce a 3D cell construct. For instance myoblasts were cultured in a micropatterned surface to tailor cell alignment before their transfer to biodegradable hydrogel [40]. The cells developed in the hydrogel and the intracellular arrangement mirrored the native muscle tissue. Natural as well as synthetic polymer-based hydrogels were researched for tendon regeneration [38,41]. Poly(ethylene glycol) (PEG)-

based hydrogels were developed to deliver marrow stromal cells for tendinopathy [41]. However, PEG-based hydrogels are known to elicit FBR [42]. FBR responses commence by protein adsorption to the scaffold leading to the adhesion of immune cells such as macrophages and foreign body giant cells (FBGC). Subsequently fibroblasts are escorted to the scaffold and collagen is deposited, which progresses to fibrosis and chronic inflammation. In addition, the adsorption of inflammation-induced protein to PEG-based hydrogels may also contribute to FBR [43]. PEG-based hydrogels are susceptible hydrolysis and enzymatic degradation leading to the generation of acrylic acid and even sometimes undergo oxidative degradation producing a cascade of radical species [42]. All these contribute to the inflammation and FBR to PEG-based hydrogels.

The injectable hydrogel approach is suitable for small tendon defects. They can efficiently fill the irregular spaces of injured tissue and circumvent the need for a surgical procedure [44]. In addition, they can function as sealants and also prevent the formation of adhesion sites [45]. Hydrogel technology can be tailored for sustained delivery of growth factors to stimulate the healing of small tissue defects [44]. However, the base materials used to produce hydrogel systems have to be thoroughly investigated for their influence in the host immune response.

27.3.2 Natural biomaterials for large tissue repairs

27.3.2.1 *Collagen*

Collagen 1, being the main component of tendon tissue, has been widely used to develop biomaterials for tendon regeneration. Collagen is fabricated in different physical forms such as sponges, biotextile fibers, and even blended with synthetic polymers to improve the mechanical stiffness [3]. Synthetic collagen fascicle scaffolds were derived from collagen 1 and PEG. The scaffold mimicked the microarchitecture of tendon tissue, and promoted the infiltration and adhesion of white blood cells and tendon cell, expressing tendon markers in vitro [46]. Large Achilles tendon defects in rabbits were treated with bioscaffold fabricated with collagen 1, bovine platelet gel, and polydioxanone. The scaffold improved the tendon healing by triggering inflammation and the progression to fibroplasia formation. Therefore this enhanced higher collagen production by the cells. In addition, tendon water uptake was boosted with decreased peritendinous adhesions [47]. Collagen-based scaffolds have also been used for muscle tissue engineering. In one study, cross-linked atelocollagen sponge was used to treat muscle defect in rabbit thigh [48]. The collagen sponge displayed a high number of large regenerating myofibers as compared to untreated controls. In another study, collagen scaffolds in conjunction with electrical stimulation were used to expand muscle precursor cells [49].

Immune responses to collagen-based biomaterials are attributed to the noncollagenous counterparts and crosslinking treatments [50]. Collagen is perceived as nonimmunogenic [50]. Although it is known to interact with antibodies, it is identified as a weak antigen [51]. An antigen can elicit a plethora of immune reaction cascades that are usually a complex interplay between humoral (antibody-based) and cell-mediated responses. In animals, immune responses to collagen were found to be both T-cell dependent [52] and T-cell independent [53], depending on the donor or recipient species. Antibodies to bovine

collagen were detected in the sera of human subjects injected with Zyderm collagen implant, but neither did the antibodies cross react with human collagen nor produce circulating immune complexes [54]. However, 3% of the injected population developed immunological reactions such as swelling, tenderness, and erythema, etc. The implant was modified by glutaraldehyde crosslinking, which lowered the immunogenicity of the implant [55]. Antibodies to bovine collagen were also observed in the sera of healthy individuals and were linked to the dietary intake of beef [54]. The preexisting hypersensitivity towards collagen may lead to collagen allergy post operation. In some cases, inflammation and granuloma formation were observed which usually subsided within a few months [50]. Prior to treatment, the patients are usually recommended to undergo two skin tests to assess the risk of developing a hypersensitivity reaction to collagen [56]. There have been raising concerns over development of collagen-induced autoimmunity diseases such as arthritis [50]. Unlike collagen type I and III, collagen type II and XI are known to induce arthritis. There is no study that shows the presence of collagen type II in a biomaterial will cause autoimmune reactions, but equally there is no evidence to disprove this concern.

27.3.2.2 Silk

High mechanical strength and flexibility are indispensable for tendon reconstruction. Tensile strength of about 150 MPa is needed for the tendon biomaterials, which is lacking in processed collagen as the tensile strength is usually below 10 MPa [57]. On the other hand, the tensile strength of silk fibers extracted from silkworm *Bombyx mori* can reach up to 690 MPa [57]. Silk biomaterials can be manufactured in different physical forms, such as films, sponges, cords, fiber mesh, hydrogels, and knitted nets [58].

Knitted and braided scaffolds provide superior mechanical strength as compared to sponges and hydrogels. But when it comes to cell attachment, sponges and hydrogels perform better. Knitted silk/collagen scaffolds subjected to mechanical stimulation encouraged differentiation of human embryonic stem cells—derived MSCs into tenocyte lineage [59]. In another study, combined silk scaffolds were produced by combining both sponges and knitted fibers of silk. Silk sponge supported cell adhesion and development, whereas, knitted silk fibers provided mechanical strength to the scaffold [60]. Silk has also been investigated for muscle tissue engineering capabilities. Since silk lacks electroactivity, the polymer is blended with electroactive melanin or water-soluble conductive poly(aniline-co-N-(4-sulfophenyl) aniline) to promote myogenic differentiation of cells [61,62].

Silk has been widely used as suture material. Silk extracted from *B. mori* predominantly comprises two types of protein, namely, fibroin and sericin. Sericin functions like a glue to hold the fibers in place. Silk-based sutures comprising both fibroin and sericin induced severe inflammatory responses in patients [63]. The cause of the inflammation was unclear; however, detailed investigations concluded that the presence of fibroin and sericin together in its native form can induce allergic response [64]. But when fibroin or sericin is used separately, no adverse inflammatory response was reported [65] [64]. Silk fibroin (SF) is widely researched for musculoskeletal grafts. It is important for a biomaterial not to induce adaptive immune response, but native forms of fibroin–sericin complex proteins triggered the production of adaptive immune antibodies [66]. Innate inflammatory responses can be subdivided as mild and severe responses. Mild inflammation can be beneficial as it promotes wound healing. Innate inflammatory responses can be acute to

chronic, this response mainly involves recruitment of macrophages. SF processed by hexafluoro-2-propanol or formic acid led to infiltration of activated macrophages in implant-tissue interface in vivo animal models [67] [68]. This acute immune response was observed in the initial 7–14 days of implantation, and the effects dissipated subsequently. Sometimes the innate immune responses can be prolonged to few months or even years, which is usually indicated by the presence of FBGC. A few investigations pointed out the formation of FBGC in SF-based biomaterials implanted in different tissues such as bone defects, fascial defects, and cervix [68] [69] [70]. The numbers of FBGC reduced after fibrotic tissue formation [71]. In another study, porous SF scaffolds displayed the presence of granulation tissue with infiltration of FBGC and lymphocytes at 4 weeks post implantation [72]. At 8 weeks, FBGC and lymphocyte numbers were significantly decreased, but granulation tissue was still present. The induction of immune responses by silk-based biomaterials depends upon the site of implantation, degradation rate, and scaffold fabrication. More research is needed to elucidate the immune reaction cascades triggered by silk-based biomaterials or modify the implants to modulate immune responses according to the clinical need.

27.3.3 Synthetic materials for large tissue repairs

Synthetic materials are widely used in tissue engineering mainly because of their flexibility and tunable mechanical properties [3]. Especially the polyester-based polymers, namely, polyglycolic acid (PGA), poly(lactic acid) (PLA), poly(caprolactone) (PCL), and their copolymer poly[(lactic acid)-*co*-(glycolic acid)] (PLGA) have garnered popularity because of their biodegradability and tunable mechanical properties. These polymers have also been approved by the FDA for specific clinical applications [73]. Electrospun fibers of PLGA support differentiation of C2C12 murine myoblasts [74]. The aligned fibers also promote elongation, alignment, and adhesion of the muscle cells. In another study, electrospun fibers of PCL/PLGA composite were investigated for myogenic differentiation [75]. Scaffolds with higher PLGA proportions displayed enhanced cell confluence and development. The cell differentiation was further improved with the incorporation of decorin, a myogenic proteoglycan in the scaffold. Synthetic polymers were also explored for tendon tissue engineering. Mouse skeletal muscle-derived cells (MDCs) laden PGA fibers were compared with tenocytes to engineer tendon graft. MDCs produced encapsulated scaffolds and produced higher collagen content and stronger tissue as compared to tenocytes [76]. In another study, PGA/PLA composite scaffolds were produced for tendon engineering [77]. The inner compartments were mainly composed of unwoven PGA fibers which were surrounded by an outer layer of net knitted PGA/PLA fibers. Autologous ADSCs were cultured on these scaffolds for 5 weeks before implantation in rabbits' Achilles tendon defect. The tissue formation was compared with cell-free PGA/PLA scaffolds. In the cell laden PGA/PLA scaffolds, the collagen was appropriately aligned and tendon formed was similar to the normal healthy tissue. Moreover, scaffolds were completely degraded by week 45. However, the control scaffolds (PGA/PLA scaffolds without cells) presented irregularly arranged collagen fibers with fibrotic tissue formations. Moreover, the scaffold–tissue interface had inflammatory cells at 42 weeks. In another study, 2D and 3D

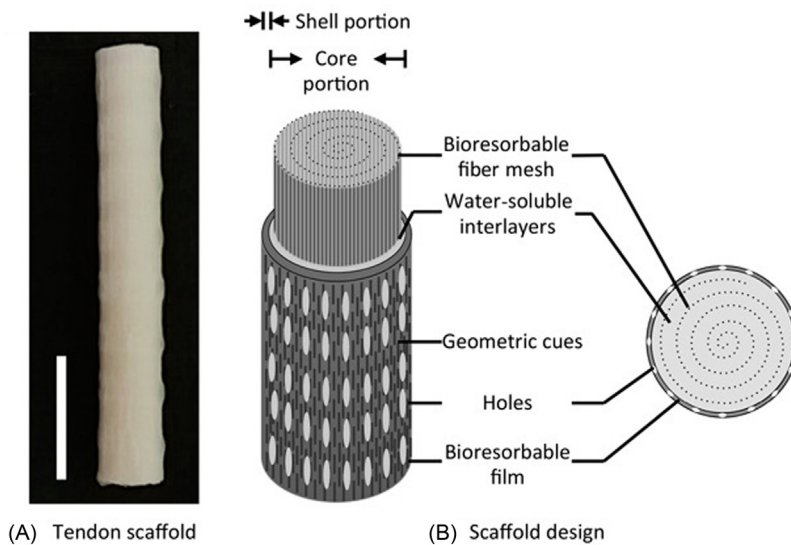


FIGURE 27.2 (A) Gross appearance of multi-layered PCL tendon scaffold. (B) Diagrammatic representation of the concentrically arranged PCL core and shell sections of the tendon graft [79]. PCL, Poly(caprolactone). Source: Distributed under a Creative Commons Attribution License 4.0.

electrospun fibers of PCL were produced for tendon repair, the graft promoted the adhesion and maturation of tendon fibroblasts [78]. The cells were arranged parallel to the scaffold's fiber alignment mimicking the native tendon tissue architecture. Moreover, the cell responses on PCL graft were comparable to autograft controls in the murine model. PCL fabrications mimicking the natural tissue anisotropy and mechanical strength have been designed (Fig. 27.2) [79]. In this novel application, the scaffold core was composed of PCL electrospun nanofibers arranged concentrically, which were wrapped by an outer layer of PCL film with elongated laser induced pores. The structure was highly porous allowing the development of tenocytes, yet had superior mechanical strength because of the intricate arrangement of the PCL fibers. Well-defined cell alignment was observed both in *in vitro* and in *in vivo* micro pig model.

The scaffolds were biocompatible and mechanically robust, as of 1 month postsurgery, animals displayed normal locomotion. Tissues were harvested after 3 months displayed neotissue formation with progressive maturation phases (Fig. 27.3). Some of these synthetic polymers were even used in conjunction with natural polymers such as collagen and silk. Combinations of PCL and SF were used to produce scaffolds for tendon tissue engineering [80].

Although these synthetic polymers are considered to be biocompatible and noncytotoxic, inflammatory responses have been reported for these polyester-based polymers. For instance, the PEG rods used to treat unimalleolar or bimalleolar fractures presented inflammatory response in human subjects. Biopsy specimens' examinations revealed the infiltration of neutrophils and implant debris being phagocytosed by FBG cells [81]. Likewise, other reports pointed the FBR formation and inflammations in patients because of the usage of biodegradable lactide-glycolide copolymer rods to treat bone fractures [82]. The inflammation is mainly attributed to the degradation products of the polyester-based polymers which are acidic in nature. Polymer such as PGA degrades to produce glycolic

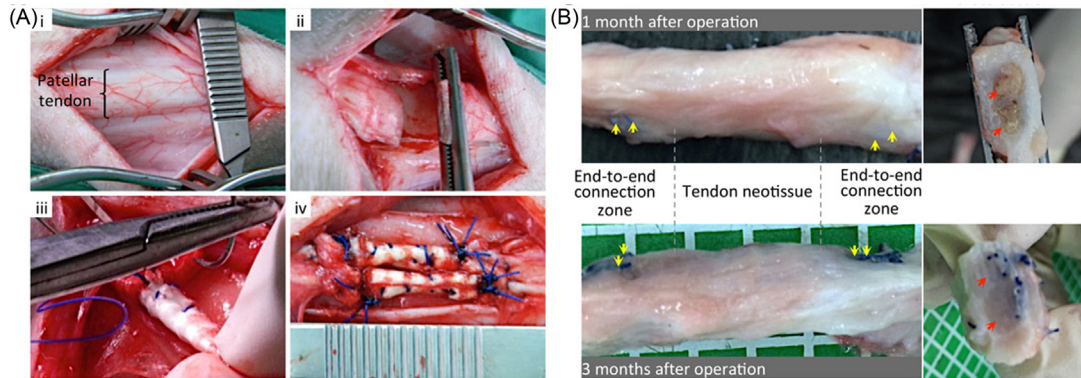


FIGURE 27.3 (A) Tendon tissue reconstruction in vivo. (i) Patellar tendon of mini pig. (ii) Tendon tissue defect is created (2 cm). (iii and iv) PCL tendon graft sutured to bridge the gap of cut ends of the tendon tissue. (B) Gross appearance of neotendon construct harvested 1- and 3-month postsurgery. The yellow arrows depict surgical line and the red arrows indicate the implanted scaffold [79]. PCL, Poly(caprolactone). Source: *Distributed under a Creative Commons Attribution License 4.0*.

acid, which can be excreted by the body as urine or can transform to glycine and subsequently to pyruvic acid which then enters and gets metabolized via tricarboxylic acid cycle [73]. Although the products can be completely metabolized, the graft placed in poorly vascularized tissues may cause undesirable accumulation of acidic byproducts. The acidic milieu thus created will fuel successive degradation of the polymer scaffold, leading to tissue inflammation [83]. Degraded products of PGA implanted in mice lead to neutrophil infiltration and the inflammation. Tissue inflammation was triggered by activation of the classic complement pathway mediated by C5a generation [84]. PCL and PLGA were implanted in mice subcutaneously portrayed distinct biological responses [85]. PLGA degraded at a much faster rate as compared to PCL creating an acidic microenvironment, which reduced infiltration of angiogenic as well as inflammatory cells to PLGA as compared to PCL. Hence, degradation rate of the polyester polymers, location of implantation, and physiochemical properties determine the biological responses in the host body. Meticulous investigations are needed to understand the interactions of polymers with the host tissue, in order to establish appropriate strategies for tissue engineering.

27.4 Conclusion

Until now, the treatment for tendon/muscle tears and ruptures are still suboptimal. It is still plagued by inferior tissue regeneration, while not forgetting about the long-term complications and morbidity on patients. The fundamental pathophysiology that underlies tendon/muscle tissue damage has to be addressed. In this chapter, the recent developments of therapeutic approaches to repair tendon/muscle tissue have been highlighted, with a specific interest in the modulation of immune reactions. Yet, the clinical need to reciprocate signaling cascade for native tendon tissue production is still unmet. Localized delivery of genes and proteins using an appropriate biomaterial may yield better results

rather than adopting one single approach. Even so, further research is required to establish a suitable strategy and formulation for scalability, biocompatibility, nonimmunogenicity, and bioactivity. The eventual goal is to have *de novo* tissue regeneration mirroring native tendon properties instead of the current inferior scar tissue secondary to repair.

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Pulmonary system responses to biomaterials

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28.1 Introduction

A number of strategies have been used over the past few decades to treat lung diseases. Lung transplantation was one of the most common therapeutic choices, however its disadvantages outweigh the advantages. For example, lack of adequate donor organs, risk of problems with coagulation, tuberculosis, diabetes, and body rejection of the new lung can be mentioned as limitations of this method. In contrast, mechanical ventilation and extracorporeal membrane oxygenation, which are the solutions for patients with acute end-stage lung disease, may result in injury due to excessive airway expansion, respiratory muscle atrophy, and too much compression [1–4]. The production of novel functional replacement tissues using tissue-engineering methods is a possible solution to the problem created by organ shortages. Tissue engineering has shown great promise for a number of tissues including bone, cartilage, liver, and pancreas. However, lung tissue engineering has not advanced as significantly, with only a few published reports focusing on the development of airway epithelial cells on synthetic polymer scaffolds. A key element in recent developments in tissue engineering and biomaterials research is the design of different types of scaffolds. Biocompatibility, degradation characteristics mechanical, chemical, and increasingly biological properties are the key criteria for the use of artificial biomaterials. Advantages of applying nanotechnology for drug delivery utilization in through pulmonary and nasal routes have shown targeting targeted drug delivery, increased of bioavailability, increase of high efficiency, reduction of bio-distribution, less toxicity and more patient comfort [4,5]. This chapter investigated multiple diseases like pneumonia, asthma, lung cancer and other respiratory diseases. Also, it discussed the biomaterials

utilized as drug carriers or tissue scaffolds in respiratory tract. Most of the studies assessed bioavailability, absorption, and biocompatibility of biomaterials. Targeted-drug delivery of lungs and engineered-tissue constructions are mostly made of polymers. Thus, here we mainly focused on synthetic and natural polymers role in therapeutic methods.

28.2 Synthetic biomaterials and their applications in pulmonary administration

For the last decade synthetic materials have been used for a great variety of lung tissue engineering and regenerative medicine applications. They offer several advantages over the natural materials including improved chemical resistance, tunability of their properties, and mechanical durability [3]. Initially, inert materials were used as implants which mainly provided the replacement of mechanical function. A wide range of biomaterials from metals and ceramics to polymers have been used for different regenerative medicine applications. However, pulmonary administration metals and ceramics are rarely utilized and most of studies are around polymeric structures [1–3]. Saturated poly(hydroxyl esters), including poly(lactic acid), poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), and poly(caprolactone) are the most widely used synthetic biopolymers in tissue engineering [3]. Several key factors should be considered to use these materials for pulmonary administration including degradation features, biocompatibility, biological, mechanical, and chemical properties [4]. Biopolymers specifically play a crucial role in soft tissue repair. The factor, which makes them more attractive for soft tissue engineering, is their flexibility terms of chemical properties [e.g., degradation rates, surface chemistry, glass transition temperature (T_g)] which are essential for biomimicked structures. Therefore, their properties can be tailored for a specific host response [2,3] (Fig. 28.1).

28.2.1 Poly(ethylene terephthalate)

Poly(ethylene terephthalate) (PET) is a nondegradable thermoplastic polymer, first commercialized under the name Dracon [4]. This semicrystalline polymer is an FDA approved, biocompatible material which has been used for soft tissue repair and large blood vessels. PET is a relatively resistant material to most gases and liquids, and this property can be improved by either increasing its crystallinity or by copolymerizing it with other monomers [5]. Because of its crystalline nature PET does not absorb water and it is also resistant to many common solvents. Other medical uses of this polymer include axillofemoral bypass [6], rhinoplasty [7], and ligament reconstruction [8], particularly in the form of woven/knitted structures. However, this polymer has several drawbacks in surgery uses. For instance, when the shape of material is important for a specific function, PET is not a suitable choice because it may undergo permanent deformation or creep under constant loads [2].

28.2.2 Poly(tetrafluoroethylene)

Poly(tetrafluoroethylene) (PTFE), commonly known as Teflon, is a thermoplastic crystalline synthetic and nondegradable polymer which is highly hydrophobic and resistant to

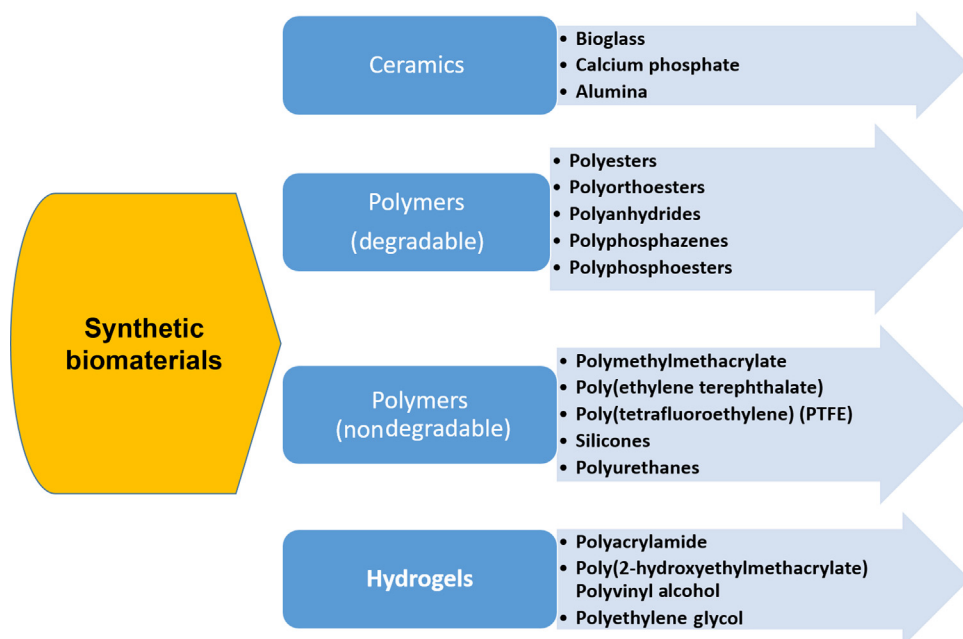


FIGURE 28.1 Examples of common synthetic biomaterials suitable for pulmonary administration. [2].

usual chemical solvents. This polymer also has a high T_g value (between 211°C and 297°C) and is resistant to thermal treatments [4]. Teflon has been used in several medical applications because of its suitable properties such as inertness, biocompatibility, and high mechanical properties. The presence of strong carbon-fluoride bonds and the absence of virtual branching results in an inert material with a Young's module around 500 and tensile strength between 14 and 23 MPa. However, Teflon may undergo creep and cause problems in medical applications [2]. Some of the PTFE applications either in woven form or injected form include artery replacement [9], synthetic ligament prostheses (Goretex) for congenital diaphragmatic repair [10], vocal-fold augmentation [11], and vesicoureteric reflux treatment [12].

28.2.3 Poly(glycolic acid)

PGA is a crystalline polymer that can be synthesized by different methods to obtain a variety of molecular weights [13]. This polymer has a tensile modulus around 7 GPa, although its mechanical properties may decrease over time due to its degradation. PGA is not very stable in aqueous environments and it would degrade in a few weeks [2]. This property of PGA is beneficial for a few applications, usually for creating temporary structures. In some in vivo studies immune responses are also reported [14]. A scientific study in lung regeneration has reported the promotion of alveolar tissue growth after seeding lung progenitor cells onto PGA scaffolds. After the seeding, PGA expressed an excellent environment for culture of specific cell types in vitro, although after implantation the

immunocompetent host induced a foreign body response that caused difficulties for the development of the lung tissue and resulted in an inflammatory reaction. According to the outcomes, it is understandable that selecting the right material for lung regeneration is very important and complicated [15,16].

28.2.4 Polyvinyl alcohol

Polyvinyl alcohol (PVA) is a biodegradable and biocompatible polymer. Using different molecular weights, polymer concentrations and cross-linker variety of PVAs with different degradation rates can be produced [17]. PVA in aqueous media will be transformed into a gel form and its stability can be achieved by freeze–thawing [18], covalent cross-linking, (e.g., glutaraldehyde [19] and gamma irradiation [2,20]). PVAs interesting features including preventing of bacterial infections and granuloma formation make it very popular for regenerative medicine applications. These properties are caused by its resistance to cell adhesion and protein absorption and results in usage of this material in eye wetting drops, contact lenses, wound dressings, and surgical sponges [21]. In addition, copolymerizing PVA and blending it with other polymers creates a variety of hydrogels with different characteristics. Each may be suitable for several applications [2].

28.2.5 Polyethylene glycol

Poly (ethylene glycol) (PEG) is a highly biocompatible polyester that is tremendously used in biomedical applications due to its favorable properties. PEG is a low-toxic and nonimmunogenicity, low weight polymer that is soluble in aqueous solutions and organic solvents. PEG properties make it a proper choice for surface modification of biomaterials, particles, and micelles for active molecule transport, and for chemical and physical hydrogels [1]. The ability of this polymer, which can be dissolved in both aqueous and organic solvents, makes it suitable for end-group derivatization and chemical conjugation to a variety of biological molecules such as polypeptides, polysaccharides, polynucleotides, and small organic molecules under mild physiological conditions [1,22]. Docetaxel-loaded PEG-albumin NPs (PEG-DANPs) were used for nonsmall cell lung cancer (NSCLC) therapy and it was also compared with Docetaxel (Aisu) and DANPs which were commercially available. The results indicated that time and dosage affected the efficiency of the PEG-DANPs. Tumor growth was reduced considerably along with the metastases in the livers of NSCLC-bearing nude mice, and it also showed less weight loss compared with the commercialized products. In conclusion, since polyethylene glycol (PEG)-DANPs had fewer side effects and also greater antitumor and metastases activity, they are considered as a potential treatment for NSCLC [23].

28.3 Synthetic biomaterials for drug delivery in lungs

Biomaterials, especially polymers have been widely used as nanocarriers in the past few years. Nanogels, nanoparticles, micelles, nanospheres, and nanofibers are examples of usage

of these materials in the field of tissue engineering [24,25]. Biopolymers play an important role in drug delivery applications because of their suitable properties. Biopolymers are amazing for drug delivery based on their appropriate biocompatibility, adaptability to extra cellular matrix (ECM) condition, biodegradability, and possibility of loading several drugs, peptides, cells, growth factors, and antibodies. They are also sustainable in releasing the loaded agents [3,26]. In drug delivery, usually a biocompatible substrate is needed for the encapsulation of therapies agents along with polymeric nanocarriers [24]. A great variety of natural and synthetic biopolymers can be used for lung regeneration. Synthetic polymers such as PLGA, poly-*N*-hydroxy-ethyl-acrylamide (PHEA), poly-ethylenimine (PEI), and PEG are also utilized for lung drug delivery or tissue engineering applications [23]. In contrast with natural polymers, synthetic polymers are more interesting materials as nanocarriers due to their more controllable properties [27]. According to the block structure, molecular weights, and degradable linkages, synthetic polymers can create different forms and types [16]. However, these polymers may have poorer biological activities which can be modified using bioactive elements [28]. Among the synthetic biopolymers that have been used in nanocarriers, PEG, PLGA, PHEA, PEI, and paclimer are currently the most common used polymers [29,30]. Many of the synthetic biopolymers are used for the regeneration of lung defects. It has been reported that synthesizing PLGANPs containing mesenchymal stem cells and doxorubicin could be an effective treatment for pulmonary melanoma metastases [31]. A sustained release system was created by He et al. using folic acid (FA)-PEG-PLGA for lung regeneration. Their results indicated a biocompatible system with no sign of blood clotting or complement situation. They have reported that these synthetic nanoparticles are promising candidates for lung cancer therapy since the codelivery of drugs highly encouraged cancer cell apoptosis and cell cycle hindrance [29]. Another scientific study reported about the ability of capsulation of chemotherapeutic mediators and small interfering RNA (siRNA) in a synthesized porous silicon-based micro/nanocomposite (MNC). Another study has reported a successful usage of a porous silicon-based MNC for delivering chemotherapeutic agents and siRNA to the lungs after intravenous injection. The silicon microparticle pores were filled with siRNA-containing liposomes B-Raf proto-oncogene serine/threonine kinase (BRAF), while the surface was filled with docetaxel-encapsulated polymeric nanoparticles. In contrast with combination therapy of liposomes and polymers, the MNC demonstrates superior therapeutic efficacy and increased accumulation in metastatic melanoma lesions in the lungs. According to the results, it can be concluded that the MNC has the potential to be used as an active delivery vehicle for enriching several therapeutic agents in the lungs [32].

28.4 Uses of synthetic biomaterials in lung tissue engineering

Great advances have been made in tissue engineering for the past 100 years. Many efforts have been made based on seeded stem cells in an avascular scaffold. With the development of technologies, performed vascular scaffold can be made with several advanced methods. The lungs are the primary organs of the respiratory system and several defects may cause fatal lung diseases which need to be treated carefully [33,34]. Some of the advancements in the field of tissue engineering and experiments for lung regeneration are reviewed in the

following text. A scaffold for lung regeneration requires a vascular network which transfers oxygen efficiently to the parenchymal chamber by using this network. Efficient transfer of oxygen and nutrients is a very important issue that needs to be considered carefully [35,36]. Basically, the scaffold is a thin porous membrane that separated the vascular network from an adjacent alveolar chamber, which has continuous oxygen flow. The exchange of oxygen and carbon dioxide happens between the blood and alveolar chamber across the membrane. An important factor for designing the scaffold is high surface area for gas exchange [35]. The scaffold with this design which is scaled for the treatment of a young adult, would result in a lung scaffold with 2.8 m² of surface area and oxygen transfer of 250 mL/min and carbon dioxide transfer of 265 mL/min. This work simply shows the feasibility of achieving gas transfer through engineered lung scaffold [35]. Silicone or other traditional absorbable materials have been widely used for creating lung scaffolds which resulted in effective constructs. Yet major mass of the polymers per unit of tissue is needed, thus the thickness of the scaffolds would be great for in vivo implantation [35]. In recent years there has been a great advancement in synthesizing different natural or synthetic materials for many medical application including lung regeneration [23,34,37,38]. Lung regeneration is currently a very important issue because of the high number of mortalities caused by lung disorders [37,39]. Tissue engineering and regenerative medicine strategies are currently the most promising methods for lung repair. Many studies are based on utilizing the xenogenic scaffolds to decellularize the lungs of big animals such as pigs [40,41], although to prevent the transmission of zoonosis strict and careful decellularization and also precise analysis of the donor tissue has to be ensured [42]. Several reports have shown that utilizing metals, natural or synthetic polymers containing suitable cells and growth factors results in a successful lung repair [43,44]. These scaffolds have many advantages in comparison with decellularized tissues. Being nonimmunogenic and a quick manufacturing process could be mentioned as its great benefits [41,45]. Petersen et al. have created a scaffold made of a lung extracellular matrix and culture pulmonary epithelium, and vascular endothelium into the scaffold using a bioreactor. The results indicated a great hierarchical organization created with the seeded epithelium within the matrix. In addition, the vascular compartment was repopulated efficiently by the seeded endothelial cells [45]. In other similar study, Joaquin Cortiella and his team seeded the adult-derived lung progenitor cells (SLPC) into a PGA and pluronic F-127 engineered scaffold for lung regeneration. After in vivo investigation, lung-specific markers for Clara cells, pneumocytes, and respiratory epithelium were expressed and organized into recognizable pulmonary structures. As mentioned in before in this chapter, usage of PGA resulted in a foreign body response that altered the integrity of the developing lung tissue. The study highlighted the importance of identifying and characterizing somatic stem cells and the need for scaffold-based approaches considering the suitable biomaterial to stem cell sources for tissue engineering [15]. Specific key factors should be taken into account when lung scaffolds are designing and mimicking the lung structure, the morphology, and mechanical properties (flexibility, compressibility, and stretchability). This includes suitable cells, growth factors and blood vessels, blood-air barrier, in conjunction with functional alveolar epithelial and microvascular endothelial cells, creating a high surface area for gas exchange, a perfusable microvasculature for fighting against thrombosis, using nonimmunogenic materials, preferably using nanoscale networks for better ECM mimicry [23,46–48].

28.5 Natural biomaterials for pulmonary applications

Natural polymers have been successfully utilized as drug carriers for various tissues including bone, cartilage, lungs, etc. [49]. The rate of drug release from these materials is controlled by breakdown of the structure and also, the products of their breakdown such as amino acids have favorable effects on cells and tissues to induce tissue regeneration and remodeling [50]. The lung is a good recipient of drugs owing to its large surface area, prevention of first-pass metabolism, and fast actions which facilitate drug absorption [51]. Hence, natural materials are potential candidates for lung drug delivery due to possibilities of reduction in drug dosage due to synergistic action of the drug and carrier [52].

28.5.1 Albumin-based biomaterials

Albumin is the most abundant protein found in human serum [53]. Other sources such as milk, egg whites, various plants, and animal tissues make this protein suitable for lung tissue engineering by cause of biodegradation, cheapness, and plentifulness [54]. The most common types of albumin applied in tissue engineering include human serum albumin, porcine serum albumin, bovine serum albumin, and ovalbumin [55]. Naturally, the presence of albumin in the lungs is utilized for metabolic processes and endogenous transportation and also, reduces the concerns about toxicity [56]. In a study performed by Woods et al. in vivo biocompatibility and clearance of albumin nanoparticles for pulmonary drug delivery were examined. The results determined high biocompatibility of a wide dosage range and mild inflammation only at highest dosages. Furthermore, it was shown that albumin nanoparticles were cleared from the lungs in a slower manner compared to tantamount dosage of albumin solution and retained in the lungs longer [57]. In another study, Chaurasiya et al. investigated the effect of the size of bovine serum albumin microparticles loaded with paclitaxel on the treatment of lung cancer. Microparticles with different sizes converted to a dry powder with a uniform size with the aim of examining in vivo biodistribution and maintenance of the drug in the lungs. The results revealed that the desired delivery system demonstrated no systemic toxicities and although the powder had a uniform particle size for inhalation, microparticles with larger sizes exhibited a more sustainable release and the drug lasted longer in the lungs [58].

28.5.2 Derivatives from silk

Silk as a natural polymer has been utilized over the decades for sutures and other biomedical applications [59]. Most of the studies in this field are dedicated to silk fibroin (SF) of *Bombyx mori* cocoons produced by silkworms due to higher quality and more production of fibers [60]. Excellent mechanical properties, biocompatibility, and biodegradation made SF a promising biopolymer for biomedical and drug delivery applications [61]. A wide range of SF biomaterials such as hydrogels, fibers, films, and particles are used as platforms for drug delivery [62]. Diverse polymeric carriers have been applied for delivery of drugs and therapeutics to the lungs and among these polymers, SF is a potential candidate because of its characteristics [63]. Different studies investigated silk-based drug

carriers for the lungs. In a study by Kim et al. spray-freeze-dried SF particles were prepared for delivery of cisplatin to the lungs through the airways as a dried powder inhaler. Cytocompatibility of SF particles versus A549 human lung epithelial cell line was confirmed [64]. In another study, Mottaghitlab et al. evaluated the effect of SF nanoparticles containing gemcitabine in a mouse model after induction of lung tumor by Lewis lung carcinoma (LL/2) cells. The delivery system demonstrated higher potential of targeted delivery in comparison with other control groups [65]. Wang et al. obtained SF peptide (SFP) through additional hydrolysis and degradation by enzyme. They investigated the anticancer effect of SFP on human lung cancer A549 and H460 cells. The results demonstrated that the designed system inhibited the proliferation of the two cell lines. Moreover, SFP effectively affected cancer cells and did not reveal toxicity for normal lung cells for the reason that normal and cancer cells differ in genomic stability [66].

28.5.3 Chitosan and its derivatives

Chitosan is a linear polycationic polysaccharide with unique properties such as biocompatibility, biodegradation, and the potential of encapsulating hydrophobic drugs. As a carrier for drugs and therapeutics, chitosan is able to control drug release and enhance the dissolution of low soluble drugs [67,68]. The cationic nature of chitosan facilitates the interaction of positive charges of amino groups with negative charges of mucosa membranes. This procedure hinders the elimination of drugs by cilia and decreases the clearance of drugs [68]. Furthermore, chitosan is capable of opening the junctions between cells and the transportation of the drugs in epithelial tissues will increase [69]. Also, absorption and bioavailability of the drugs will promote which makes chitosan a suitable choice for pulmonary drug delivery. Cunha et al. prepared inhalable chitosan microparticles through spray-drying for lung delivery of isoniazid and rifabutin with the aim of treating tuberculosis. Cytotoxicity assays revealed that human alveolar epithelial (A549) cells are tolerant of the delivery system. Chitosan microparticles induced macrophage activation, and macrophage uptake was observed up to 99.9%. Moreover, antibacterial activity of microparticles against *M. bovis* suggested the potential of the desired system for lung tuberculosis therapy [70]. In another study by Vijayakurup et al., curcumin-loaded chitosan nanoparticles were designed to prevent benzo[*a*]pyrene-induced lung carcinogenesis. In vitro drug release of curcumin exhibited sustainable release over 180 hours and cytotoxicity in cancer cells. Furthermore, bioavailability studies in a Swiss albino mice revealed highly localized chitosan nanocurcumin in the lung in comparison with free curcumin [71].

28.5.4 Gelatin

Gelatin is a natural biopolymer with unique properties such as biocompatibility, biodegradation and multifunctionality produced through hydrolysis of animal collagen [72]. As a denatured protein it could be degraded by collagenase enzymes and demonstrates low antigenicity compared to collagen [73,74]. Due to the presence of both anionic and cationic groups in the structure of gelatin, it is capable of encapsulating both hydrophobic and hydrophilic drugs [75]. In addition, accessibility of functional groups, ease of chemical modification, and the ability to form a thermoreversible gel turn gelatin into a

potential candidate for targeted drug delivery [76]. Gelatin-based particles demonstrate many advantages for pulmonary drug delivery because of high distribution over lung epithelial cells and more availability of therapeutics to the infected cells. In a study performed by Abdelrady et al., gelatin methotrexate-loaded nanoparticles were produced by a desolvation method in order to treat lung cancer. The process of fabrication was optimized via a Box-Behnken design of experiment to prepare nanoparticles with uniform sizes suitable for cancer cell uptake. The results revealed preferential uptake of gelatin nanoparticles by lung cancer cells in comparison to macrophages. Also, cytotoxicity assays demonstrated an increment in cytotoxic activity of methotrexate compared to free drug [77]. Youngren-Ortiz et al. fabricated gemcitabine (Gem)-loaded gelatin nanocarriers (GNCs) that were cross-linked with genipin (Gem-GNCs) in order to evaluate lung anticancer efficacy. The delivery system demonstrated controlled release owing to diffusion/erosion from nanocarriers. Cell viability assay versus A549 cells that were treated with Gem-GNCs did not reach 50% cell kill after 48 hours, but after 72 hours the Gem-GNCs gained a two-fold increase in the IC₅₀ of Gem-GNC treatment in comparison with the Gem solution [78].

28.5.5 Hyaluronic acid

Hyaluronic acid (HA) is a unique and linear anionic mucopolysaccharide composed of N-acetyl glucosamine and D-glucuronic acid [79]. A large number of carboxyl and hydroxyl groups in the structure of HA facilitate chemical modification to bind bioactive agents [80]. It is biocompatible, biodegradable, and capable of combining with specific receptors on the surface of cells. Moreover, HA is a ligand of CD44 receptors which are overexpressed in many tumor cells [81]. The application of HA in drug delivery received a lot of interest due to the ability of reacting with other drugs and formation of conjugates. These conjugates have a targeted effect and controlled release. HA that is naturally present in the lungs is able to protect pulmonary elastin against inflammation [82,83]. Based on a study, utilization of HA particles for the delivery of drugs to the lungs and nose prolongs the retention time of the drug in the lungs. Cadete et al. prepared Docetaxel-loaded HA nanocapsules through a self-emulsification method. In vitro studies versus A549 lung cancer cells exhibited intracellular delivery of Docetaxel and also blank nanocapsules demonstrated very low cytotoxicity [84]. In another study performed by Mahmoudi et al., HA-based nanostructured lipid carriers (HA-NLCs) were designed to deliver apigenin to induce apoptosis in lung cancer cells. Cytotoxicity assays against A549 cells illustrated a dramatic antitumor activity. According to Fig. 28.2, the designed system induced cell death via the Nrf2 pathway and downstream target genes [85].

28.6 Conclusion

This chapter reviewed that effects of biomaterials after pulmonary administration. This chapter classified biopolymers, which are practically utilized for lung applications, into two main groups. These classifications illustrate that synthetic polymers are more

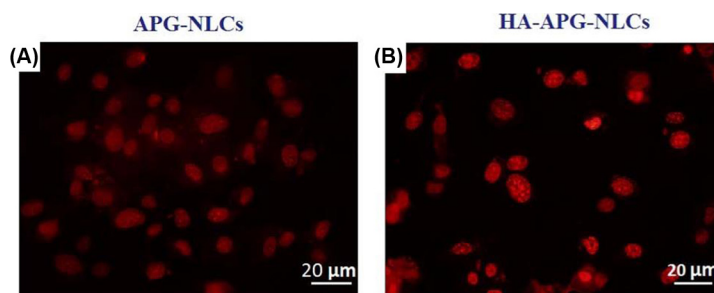


FIGURE 28.2 In vitro internalization of Rhodamine B-loaded APG-NLCs (A) and Rhodamine B-loaded hyaluronic acid-coated NLCs to enhance APG. (B) After incubation with A549 nonsmall cell lung cancer [85]. APG-NLCs, Apigenin-nanostructured lipid carriers; NLCs, nanostructured lipid carriers. Source: With permission Mahmoudi S, Ghorbani M, Sabzichi M, Ramezani F, Hamishehkar H, Samadi N. Targeted hyaluronic acid-based lipid nanoparticle for apigenin delivery to induce Nrf2-dependent apoptosis in lung cancer cells. *J Drug Deliv Sci Technol*, 2019;49:268–76.

controllable than natural polymers for precise applications. However, natural polymers are more compatible with native lung tissue. Although there are many studies dealing with biomaterials for the pulmonary system, the exact interaction mechanisms have not been fully understood yet. Research on this topic can improve the functionality of biomaterials in the pulmonary system.

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Gastrointestinal response to biomaterials

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29.1 Introduction

Gastrointestinal (GI) tract is a crucial organ for humans and other animals for converting the food to energy. This organ expels the remaining waste as feces and eliminates it from the body [1]. GI consists of very long components with elaborated functions. Consequently, there are lot of disorders and assessments for GI diseases [2]. In this chapter, we expressed the efficiency and biocompatibility of future promising studies through in vitro and in vivo trials. Targeted-GI drug delivery and biomimicked-reconstruction of GI's organs are hot topics of bioengineering projects. We assessed the effects of drug delivery and tissue engineering on important organs of GI. Also, we investigated the effects of biomaterials and drugs on GI cell. The oral route constitutes the preferred route for drug delivery [3,4]. While, oral pathway provides poor bioavailability for drugs administration, polymeric nano/micro particulate systems association helps this trouble for this route because of their interaction with the mucosal surface. The present review expressed the GI bioadhesion of nano/microparticles and their biomimicked structure for matchable behaviors in different parts of the GI tract. On the other hand, this review explained the recent studies in bioengineered-tissue for GI tract with an emphasis on cell types of exact organ, scaffold components, and mild-term and long-term results of biocompatibility.

29.2 Oral cavity and pharynx

GI tract represented an input and an output, the entrance of this tract is oral cavity and pharynx thereafter. The pharynx belongs to the upper digestive tract and its morphology and function enable normal swallowing [1]. Dysphagia is one of the most important symptoms in pharynx and oral cavity and dysphagia effected the GI disorders, for example, reflux disease may show extra oesophageal manifestations in the pharynx [2]. In this regard, Shayan et al. for the first time introduced a device for potential treatment of swallowing difficulty in dysphagia patients. They designed the first tongue prosthetic assist device (TPAD). In this study, Shayan utilized superelastic nitinol and denture acrylic resin as two main biomaterials in TPAD. Superelastic nitinol used as the dynamical backbone in TPAD and denture acrylic resin as a main fabrication segment [3]. Acrylic resin also exploits in dental and oral reconstructions and replacements based on its suitable compatibility with oral cavity and GI system [4–6]. Acrylic resin reduced the saliva antioxidant defense potential by reducing the levels of uric acid [7]. In other practical study, Sinha et al. replaced the pharyngeal defects with using acellular dermal matrix (AlloDerm) and sternocleidomastoid (SCM) muscle flap. In this study, patients underwent reconstruction of through-and-through defects of partial pharyngectomy for squamous cell carcinoma using AlloDerm graft [8]. AlloDerm is the most successful dermal replacement that is developed with collagen (COL) as a natural biomaterial [9]. AlloDerm is an acellular dermal matrix that is derived from donated human skin and used as a main segment of scaffold like facial, breast, abdominal wall, and oral reconstructions [10–12]. Sinha reinforced the AlloDerm grafts with based SCM muscle flap in clinical trials. Patients who took this replacement were followed for 20 months to assess the compatibility and contracture of graft. There was a high success rate for graft take in patients according to the results of this trial [8]. There is a famous cancer in the digesting route named hypopharyngeal cancer. Hypopharyngeal is the area where the larynx and esophagus meet. It first forms in the outer layer (epithelium) of the hypopharynx (last part of the pharynx) [13]. The next oral disease is oral cancer (OC), OC is a serious and growing problem which constitutes a huge burden on people. Statistics show growth in an OC patient in 2017 [14]. Nanotechnology-based drug delivery systems as therapeutics for cancers are often viewed as a cutting edge effective and targeted drug delivery, and OC is not consider an exception for this approach [15]. Thus, there are a lot of studies in this regard including Ayalew et al. They practically used hybrid peptides as a carrier of cancer drugs for OC. Paclitaxel (PTX) is a very potent chemotherapy drug, but its hydrophobic and low solubility characters cause trouble with administration [16]. Changing the chemical formulation modified low solubility and hydrophilicity of PTX [17]. Ayalew's novel idea was about fabricating hybrid COL-cell penetrating peptide (COL/CPP) carrier [18]. COL/CPP peptide consists of two functional segments, CPP, and COL domain. CPPs can penetrate cellular membranes and cooperate with the cellular internalization of biologically relevant cargo. The COL folding domain allows the peptide to fold into the rigid nanoparticle which increases resistance to enzymatic degradation [19]. Ayalew reported that COL/CPP peptide conjugated to PTX to form an effective drug delivery system for acute T-cell leukemia (Jurkat cells) [18]. Later, Ho et al. assessed the possibility of COL/CPP application as a potential carrier to targeted-delivery of cancer drugs to hypopharyngeal carcinoma (FaDu cells). In this study, results showed that CPP domain

enables the adsorption of the carrier on the cell surface and it is caused by presence of heparan sulfate proteoglycans on the cell surface, due to overexpression of nonfunctional heparanase. In brief, Ho introduced COL/CPP hybrid peptides as a viable carrier of PTX to FaDu cells [20]. In a similar project, hypopharyngeal cancer has been worked into another project by Jun Wang. On the other side, bone marrow mesenchymal stem cells (BMSC) represent a promising targeting vector for general tumor radionuclide therapy. Wang assessed the dynamic of distribution of BMSC in the mouse model with hypopharyngeal carcinoma. The imaging of transplanted BMSCs in a hypopharyngeal carcinoma mouse model introduced BMSC as a promising targeted-delivery carrier for hypopharyngeal cancer gene therapy. For personal use only [21].

29.3 Oesophagus

The next organ of GI system is oesophagus. It is a muscle tube and it has a very important role in connecting mouth to stomach and the correct function of oesophagus causes food and liquids that have been swallowed into the pharynx to reach the stomach [22,23]. Tissue engineering of oesophagus has been developed by various studies [24,25]. Surgical repair of esophageal injuries with autologous GI segments is often associated with dysmotility, dysphagia, and donor site morbidity [26]. Removing oesophagus muscle by surgery due to cancer therapy is an accepted treatment. However, tissue elimination resulted in thickness defect which cannot be left untreated and for this aim, tissue engineering projects retrieved the damages [27]. Algarrahi et al. worked on bilayer silk fibroin (BLSF) scaffolds for their ability to support functional restoration of damaged esophageal tissues in a rat model. Reconstruction of damaged tissues with BLSF scaffolds restored luminal esophageal caliber, enabled solid food consumption, and promoted the formation of innervated. In this study, they investigated around immunohistochemical and histomorphometric assessments of protein expression and neotissues of reconstructed tissue and control tissue for 2 months and the results showed compatibility of silk fibroin with oesophagus tissue of rats [28]. Reconstruction of oesophageal needs surgical intervention with the stomach, jejunum, or colon. In these surgical substitutions, tissue engineering is promising to be an effective regenerative procedure, but no functional solution currently exists for esophageal [29]. Chung et al. worked on a stunning method of fabricating by reinforcing the oesophagus's scaffold with three-dimensional (3D) printed ring. They embedded the omentum of rats in nanostructured scaffolds and transplanted for the repair of circumferential esophageal defects. Chung utilized a synthetic polymer polycaprolactone (PCL) as a bio-ink in this project according to overcoming source scarce, low mechanical strength, and fast degradation of natural polymers [30,31]. Chung fabricated a stainless rod with PCL rings, afterward electrospined the polymer-metal composite by PCL solution for higher mechanical properties and better porous distribution for cell migration. The whole biomaterial was put in stomach of mouse to be cultured for 2 weeks. Then, implanted the cultured biomaterial in oesophagus of mouse. After 2 weeks of culture, the graft maintained a tubular structure without any infection or adhesion. After 14 days, cell toxicity data showed acceptable toxicity. Histology reports showed several newly developed blood vessels on the outer side of the scaffold that indicated a tissue regeneration process. On

the other hand, a mechanical test consisted of strain–stress sheets expressed mechanical imitation of implant with muscle of oesophagus [32]. The cells utilized for seeding these oesophagus scaffolds range from induced pluripotent stem cells, amniotic fluid MSCs (AF-MSCs), to bone marrow-derived MSCs (BM-MSCs) and cell seeding is the other solution for oesophagus diseases [33]. In another form of oesophagus tissue engineering, Barron et al. worked on attachment and growth of swine esophageal mucosal cells on electrospun synthetic nanofiber matrices for repairing the eliminated tissue of oesophagus through surgery or cancer. They fabricated scaffold to be utilized in an ex vivo bioreactor for generation of an esophageal conduit and subsequent implantation [27]. Although, acellular scaffold implantation of synthetic materials indicated that there is epithelial and mucosal regeneration near the proximal and distal segments near the esophagus, the mid zone scaffold tends to occur in biologic materials better than synthetic materials. Barron claimed that electrospun nanofiber polyurethane (PU) matrices are a suitable synthetic substrate for esophageal regeneration. PU not only allows mucosal cell attachment, but also it shows high elasticity and strength near to native esophagus. They PU nanofiber is clearly capable of esophageal regeneration in the pig model and Barron emphasized its compatibility [34]. Oesophagus could be targeted for treatment of some upper gastrointestinal tract diseases like Avian *Trichomonas* [35]. *Trichomonas gallinae* is a flagellated protozoon that causes avian trichomoniasis. This parasite affects the upper digestive tract and various internal organs of a wide range of bird species [35]. It is of veterinary and economic importance to treat or even eliminate this parasite and the main drug for Avian *Trichomonas* is metronidazole but it has side effects [36]. Indeed, Younessnia et al. worked on a new strategy to reduce side effects of metronidazole through drug delivery in oesophagus [37]. In this study, nanocapsules of chitosan prepared and went up on cellulose nanofibrils. Nanoparticles of chitosan have been retreated by tannic acid to increase their water solubility [38]. This strategy helped to separate chitosan nanocapsules at the purpose site in oesophagus and then released their antiprotozoal drug. Reports of this study indicated this carrier could be used against *Trichomonas gallinae* because of their biocompatibility and better release yield [37]. Fig. 29.1 expressed the two possible method for promising tissue engineering of GI tract.

29.4 Stomach

The stomach is a muscular organ located on the left side of the upper abdomen. The stomach receives food from the esophagus [39]. The stomach is a crucial member of GI system and needs more consideration. Stomach cancer is the fourth most common fatal cancer in Europe [40]. In this part, we assess the stomach with three approaches of compatibility, stomach bio mimicking, tissue engineering compatibility, and drug carrier compatibility. In stomach diseases, like gastric cancer, neoplastic cells are limited to inner tissue of lamina propria located beneath the gastric epithelium. Therefore, new nanoparticulate drug delivery systems can be challenged by crossing the stomach epithelium [41,42]. The mucosa is the innermost layer of the stomach and all of the surface wall is covered by a single-layered columnar epithelium that lies under the lamina propria, which is largely composed by a rich and highly cellularized extracellular matrix (ECM), containing a varied cell population, including fibroblasts and smooth muscle [43]. Based on this

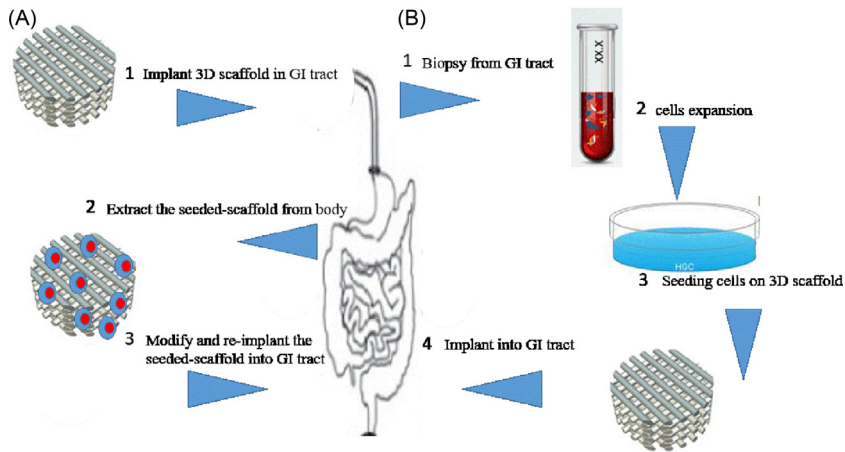


FIGURE 29.1 This schematic declared two promising methods for GI tract tissue engineering. (A) In this method, 3D scaffold implants in an organ of GI tract (due to cell types of organ) and cells migrate in free spaces of 3D scaffold. The cell-seeded scaffold extracted through surgery from GI tract, and some of extracted-scaffold properties (mechanical, chemical, or biological) were modified. Afterward, the modified-seeded-scaffold reimplants in patient's GI tract [32]. (B) The second practical method starts with extraction of biopsy tissue from the GI tract. Further, extracted cells proliferated and seeded on 3D scaffold which is previously constructed. Finally, the cell-contained scaffold implanted into the GI tract again [33]. 3D, Three-dimensional; GI, gastrointestinal.

challenges, Lourenço et al. worked on a bioengineered 3D hydrogel model that reflects the dimensionality of the gastric mucosa, provides bioactive signals to embedded cells, and provides a substrate that enables cell penetration [44]. In this study they utilized a novel 3D in vitro model of gastric mucosa, combining a human gastric epithelial cell line (MKN74), normal human stomach fibroblasts (NST20), and a bioengineered ECM-like matrix, to investigate the permeability of model nanoparticles across the stomach mucosa. Lourenço used alginate-based hydrogels tethered with a physiologically relevant density of cell-adhesion RGD ligands to mimic the 3D ECM-like microenvironment of lamina propria in gastric mucosa [45]. Low polymer concentration (1 wt.%) was used to generate soft and highly permeable alginate hydrogels. Further, stomach fibroblasts were cultured within RGD-alginate 3D matrices for 14 days, and results showed cell proliferation, protein production, and metabolic activity over time. Lourenço mimicked the stomach tissue for investigation and ex vivo trials. In conclusion, they achieved a fully compatible 3D model system of the stomach which has great potential to become routinely adopted as an evaluation platform to assist the development of new strategies for the treatment and diagnosis of gastric diseases [44]. One of the important and epidemic diseases of the stomach is gastroparesis. Gastroparesis is a disorder characterized by delayed gastric emptying of solid food in the absence of a mechanical obstruction of the stomach [46]. Gastroparesis is now recognized as part of a broader spectrum of gastric neuromuscular dysfunction that includes impaired gastric accommodation [47]. Joddar et al. investigated on the rare method for treatment of gastroparesis through MSCs delivery with gelatin–alginate hydrogels as a carrier. Mouse MSCs were seeded into alginate–gelatin that were coated with poly-L-lysine. These cell–gel constructs were placed atop stomach explants facing the

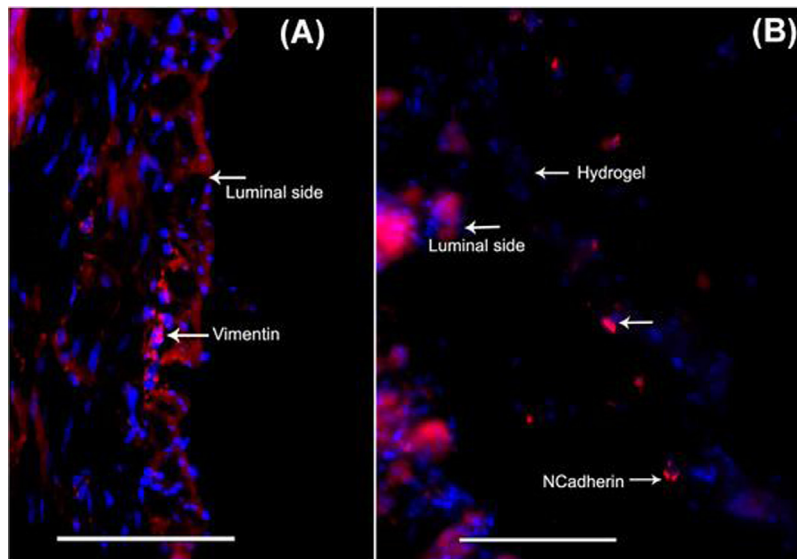


FIGURE 29.2 Delivery of MSCs delivery with gelatin–alginate hydrogels. MSC-seeded gels were placed atop the stomach lumen, the cells migrated from the gels to the luminal tissues, as shown by positively stained cells for the stem cell markers, (A) vimentin and (B) N-cadherin (as MSC indicators). Scale bar 100 μm [48]. MSCs, Mesenchymal stem cells. Reproduced with permission from Joddar B, Tasnim N, Thakur V, Kumar A, McCallum RW, Chattopadhyay M. Delivery of mesenchymal stem cells from gelatin–alginate hydrogels to stomach lumen for treatment of gastroparesis. *Bioengineering* 2018;5(1):1–14, with permission.

luminal side. MSCs grew all across the gel surface within 48 hours. When located in the lumen of the stomach, MSCs moved from the gels to the tissues. According to Fig. 29.2, MSCs delivered from biomaterial scaffold to stomach tissues, and this approach could be applied in vivo to help restore gastric function in gastroparesis. Also, reports indicated the survival, adhesion, and proliferation rates of the MSCs and other physiological improvements in the stomach wall [48]. Stomach targeted drug delivery is a difficult approach for biomaterials because of acidic environment and easy degradation in stomach [49]. Zhou et al. worked on targeted delivery microrocket for stomach cancer. Microrockets consist of inner macromolecular polyamino acids (PAA) and outer polyaspartic acid (PASP) layer, a thin iron (Fe) intermediate layer, and a core of zinc particles (Zn). The iron layer is introduced in the tubular microrocket to facilitate magnetic control. PAA exposed suitable biodegradation and biocompatibility in drug delivery systems [50]. Fe has also been used due to its biocompatibility and high safe dosage [51]. Zhou controlled the biocompatibility and biodegradation of these drug carriers. Zhou utilized a specific method for stomach drug delivery. In this method, the Zn particles are electrodeposited into PASP/Fe to powerfully propel the microrockets in an acid environment by producing hydrogen bubbles and this method has been used in previous studies [52]. Zhou developed this technique by attaching Doxorubicin (DOX) to the surface of bubble-propelled microrocket, which is a chemotherapy medication used to treat cancer. The DOX-loaded microrockets can be trapped in the gastric mucus gel layer, and slowly release concentrated

DOX payloads onto the stomach wall in acidic environment. This carrier has been tested in a mouse model and reported these microrockets as a biocompatibility, low toxicity, and a zero-waste profile [53]. One of the GI tissue engineering approaches is acellular method which is reliable in different parts of whole digest tract. Hori et al. worked on this approach with a COL sponge prepared from pig skin which was reinforced with a thin layer of polyglycolic acid (PGA) as a scaffold. They implemented the biomaterial in dogs to cover the defect on the luminal side. The biomaterial patches showed no signs of anastomotic problems and supported tissue regeneration. However, the muscle layer was not presented completely and this method needed second surgery for removing sutures [54]. In this way, Araki et al. fabricated a three-layer scaffold composed of poly(D,L-lactide) and ϵ -caprolactone, COL, and PGA nonwoven fabric and assessed it on dog. The results showed suitable wall regeneration and better biocompatibility in comparison with Hori. Despite, regeneration of the smooth muscle layer did not occur and early shrinkage of the implanted scaffold was also observed [55]. The next approach of stomach tissue engineering is fabrication of stomach. Scaffolds can be assembled using a variety of biocompatible materials and methods. 3D scaffolds are preferred as they provide better support encouraging cell adhesion and proliferation and guide the tissue regrowth mimicking the actual tissue microenvironment [56]. Maemura et al. seeded the isolated stomach epithelium organoid cells on biodegradable composite polymer tubes of PGA mesh that coated with poly-L-lactic acid. The cell-seeded polymer tubes were implanted into rats. The scaffold formed cyst-like stomach formations. In histological assessments, the fabricated-stomach showed vascularized tissue with neomucosa lining the lumen. Anastomosis between the units and native small intestine may have the potential to stimulate epithelial growth. They also found the presence of a smooth muscle layer and a well-developed epithelium which was not observed in acellular approaches [57,58].

29.5 Small intestine

The small intestine is the part of the intestines where 90% of the digestion and absorption of food occurs, the other 10% takes place in the stomach and large intestine. The main function of the small intestine is absorption of nutrients and minerals from food. Consequently, the small intestine has a crucial duty in human life [59]. On the other hand, Small intestine plays a vital role in the absorption and metabolism of oral drugs [60]. Carbohydrates are inverted in the small intestine to glucose, galactose, and fructose, and these sugars are then absorbed by enterocytes [61]. Small intestine has various disorders like bleeding [62] and infection [63]. Celiac disease is an autoimmune disease that prevents humans from eating gluten-containing foods [64]. Crohn's disease is an inflammatory bowel disease (IBD) and causes inflammation of small or large intestine [65]. Small intestine cancer is another disease of this important organ. Small intestine cancer is a rare cancer. Investigators suggested that several medical conditions may be predisposed to increased occurrence of this cancer, but otherwise its etiology is unknown [66]. The types of cancer found in the small intestine are adenocarcinoma, sarcoma, carcinoid tumors, GI stromal tumor, and lymphoma [67]. The small bowel obstruction is usually caused by scar tissue, hernia, or cancer [68]. Peptic ulcer disease (PUD) is a common disease worldwide

also known as peptic ulcer, PUD occurs as a defect in the mucosa of the stomach or duodenum which gains the muscularis mucosa. Peptide ulcer is formed as a result of inflammation that is caused by the bacteria *Helicobacter pylori* and it is a fairly common health problem [69]. This section assesses the latest update for drug delivery and tissue engineering for small intestine. Improved efficacy is attempted for drugs that are used in the treatment of gastric disorders like ulcers and *H. pylori* infections. Low concentration of the antibiotic that reaching the bacteria under the mucosa, instability of the drug in acidic environment of gastric fluid, and short residence time of the antibiotic in the stomach; controlled drug delivery systems with a prolonged residence time in the stomach have been used [70,71]. Gastroretentive dosage forms (GRDFs) are designed to be hold longer in the stomach and small intestine and release their active ingredients and thereby enable sustained and prolonged input of the drug. They can be delivered efficiently thereby maximizing their absorption and enhancing absolute bioavailability. GRDFs are investigated through in vivo bacterial clearance on a *H. pylori* infected mouse model. GRDFs expressed significant control of *H. pylori* infections and showed complete biocompatibility [72]. On the other side, tissue-engineered small intestine was generated in an autologous preclinical method. These engineered intestines were surveyed in large animals by Sala et al. [73]. In this study, short bowel syndrome has been assessed and tissue engineered small intestine was introduced as a therapy for the first time. Sala team provided a short segment of a stomach from a 6-week-old pig and loaded the multicellular clusters and organoid units in biodegradable scaffold. The scaffold consisted of PGA tubes that was coated with poly-L-lactic acid and COL type I. The cell-contain biomaterial implanted intraperitoneally in the autologous host. The implant was harvested and assessed after 7 weeks. Wonderfully, small intestine mucosa was composed of columnar epithelium with differentiated intestine cell line and subepithelial myofibroblast. In addition, cells positively responded to intestinal stem cell biomarker (double cortin and CaM Kinas-like-1) [74]. According to Fig. 29.3, histology and immunohistochemistry assesses showed complete reformation of small intestine and biocompatibility [73]. Another important trouble for small intestine is cancer. Advances in diagnosis and treatment of cancer reduced the deaths of small intestine cancer patients. Drug delivery strategies have a crucial role for saving many lives [75]. For small intestine cancer, it is better to utilize oral drug delivery methods because of their low cost, minimal invasiveness, and flexibility of various dosage [76]. Interferon alpha (IFN- α) is a chemotherapeutic drug that used in many studies in small intestine cancer due to its efficiency and nanomedicine compatibility for drug delivery [77]. Although, high dosage of IFN is vital for an efficient result in cancer therapy; however, IFN is highly toxic and the exact amount of IFN should be utilized to achieve both efficiency and biocompatibility [78]. Based on this short story, Caldorera-Moore et al. used poly (methacrylic acid-grafted-ethylene glycol) pH sensitive hydrogel nanoparticles as a carrier of IFN through oral route and they assessed the compatibility and efficiency of their novel carrier with monolayer model of human epithelial colorectal adenocarcinoma cells (Caco-2) and HT29-MTX human colon carcinoma cells which is proved previously by them [79,80]. In this study poly(methacrylic acid)-grafted-poly(ethylene glycol) methyl ether methacrylate–cotert-butylamino methacrylate [P(MAA-g-EG-cotBMA)] nanoparticles was synthesized with a specific technique and exploited as an IFN carrier for small intestine cancer. Cytopatibility studies showed the smart nanoparticles did not cause detrimental

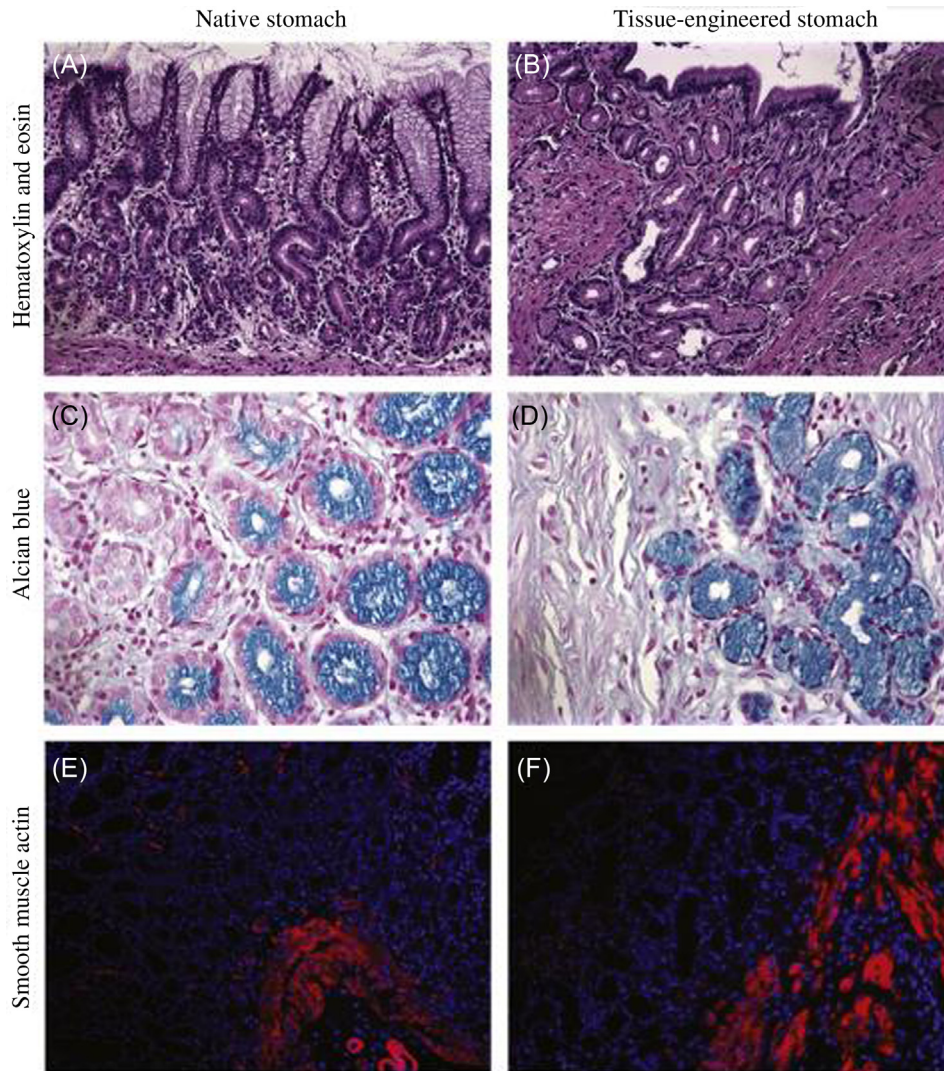


FIGURE 29.3 Tissue-engineered stomach morphology (B) was similar to the antrum of a native stomach (A). (C and D) Alcian blue staining of the mucous epithelial cells. (E and F) Immunofluorescence staining of the muscularis using an antismooth muscle actin primary antibody. These comparisons were proved successfully architectural imitating through tissue engineering [73]. Reproduced with permission from Sala FG, Kunisaki SM, Ochoa ER, Vacanti J, Grikscheit TC. Tissue-engineered small intestine and stomach form from autologous tissue in a preclinical large animal model. *J Surg Res* 2009;156:205–12 with permission.

effects to Caco-2 cells. Despite, IFN efficiently transported through thigh junction of simulated monolayer. Consequently, they introduced this carrier as an ultimate for sensitive protein chemotherapeutic agents via the oral pathway.

29.6 Large intestine

The last part of GI tract is the large intestine, colon, or large bowel [81]. The major function of the large intestine is to absorb water from the remaining indigestible food matter and transmit the useless waste material from the body [82]. In this section, drug delivery methods were used for large intestine disorders to survey biocompatibility and efficiency. Recent studies showed interest in colon-targeted drug delivery for prevalent diseases like IBDs, specifically Crohn's disease, ulcerative colitis, and colorectal cancer [83,84]. Colon pathway not only facilitated minimal side effects for various types of drugs but also, prolonged the present time of drugs in colon sites. However, toxicity and denaturing in GI system are almost the problems of colon-targeted drug delivery [85]. In this regard, disulfide cross-linked polymers are alternative carriers for colon cancer drug delivery systems. These polymers provide minimal biocompatibility and maximal bioavailability of anticancer drugs like paclitaxel (PCX), a famous drug for colon cancer [86–88]. Ayub et al. promoted novel biodegradable thiolated sodium alginate (TSA)-derived nanospheres with modified surface as an enhanced-carrier of PCX. This carrier improved the delivery of PCX to the colon site for treatment of colon cancer [89]. Cytotoxicity results expressed complete biocompatibility of nanospheres with HT-29 and CRL 1790 cell lines. Although, more than 70% of nanospheres detected in HT-29 cells, which indicated successful cellular internalization of nanospheres in the cancer cells. Colon cancer is recorded as the third cancer that causes the death in the world thus, it needs an exclusive attention [90]. In another study, El-Satar et al. investigated around the cytotoxicity of oral administrated micronized zeolites anticancer 5-fluorouracil (5-Fu) delivery systems for colon cancer treatment. Cytotoxicity of samples were tested using human colorectal adenocarcinoma cells (Caco-2 cell line) and assessed according to the mitochondrial-dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan. In conclusion, zeolite exposed a safe behavior for the Caco-2 cells while, all were of cytotoxic action when loaded with the 5-Fu drug [91]. Chemotherapy has become a more standard therapeutic approach than surgery for colon cancer [92]. With this in mind, 5-fluorouracil (5-FU) is the first chemotherapy drug that has been implemented for over four decades [93,94]. Radu et al. worked on 5-FU drug like El-Satar team but, with a different carrier. Radu utilized poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) as a nanocarrier. Within the in vitro test, 5-FU was evaluated against HT-29 human colon cancer cells in 24 hours. 5-FU-loaded PHBV significantly increased lactate dehydrogenase activity of HT-29 colon cancer cells after 24 hours and induced carcinoma cell's death. However, PHBV itself not only exerted insignificant toxic effects on cells but also successfully frustrated the highly toxic effect of 5-FU on other cells and provided convenient biocompatibility [95].

29.7 Conclusion

This chapter declared a new point of view for investigators who decided to work on GI's organ disorders. This chapter expressed the latest studies in tissue engineering and targeted-drug delivery in digestive organs, from the enter route to the exit pathway. There

are many diseases in this wide pathway; however, they need to be investigated for more details. This chapter separated these diseases by their prevalence and hazardousness to illustrate assured routes of investigation. The most recent methods and techniques studied for GI diseases suggested that the compatibility and efficiency of targeted-drugs and engineered-tissues with natural or simulated-GI tissue could play a critical role for effective treatments.

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Ocular responses to biomaterials

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30.1 Introduction to biocompatibility in the eye

Ocular diseases, such as glaucoma, cataracts, and macular degeneration affect over 70 million people worldwide, with cataracts alone leading to blindness or visual impairment in 11 and 35 million people respectively. With an aging population, the number of people affected by ocular diseases and vision loss is rising, the incidence of glaucoma (the second leading cause of blindness) was projected to reach over 79 million in 2020 from 60 million in 2010 [1]. The economic and social burden of ocular diseases is significant: recent studies estimated the healthcare system costs associated with poor vision, blindness, and resulting disability to be over 129 billion dollars in the United States and 49 billion euros in Germany [2,3]. To protect, preserve, or restore vision, medical devices such as intraocular lenses (IOLs), glaucoma shunts and novel drug delivery systems are being implanted. However, with the use of ophthalmic devices, complications such as fibrosis and inflammatory reactions impair the success of devices and reduce their functionality. Extensive research is being undertaken to develop better biomaterials.

Biocompatibility of materials plays a significant role in the success and failure of medical devices. The definition of biocompatibility has evolved over the years with the current broad but applied definition considering biocompatibility as the ability of a material to perform with an appropriate host response in a specific application [4]. From this definition, one can infer that physical, chemical, mechanical, and biological properties of a biomaterial will all contribute to the biocompatibility of a material. While chemical, mechanical, and physical properties of biomaterials are usually well characterized, a lack of understanding and ability to characterize how biomaterials interact with cells and how material properties affect biocompatibility can limit our ability to design better biocompatible materials. As cells interact with materials in a physiological environment, several biological mechanisms may be induced, resulting in cell death and/or activation as well as material degradation.

The eye is a complex biological system as it is composed of different cell types, irrigated by several physiological solutions, subjected to various mechanical stresses and which environment can also be affected by diurnal variations. Defining ocular biocompatibility is thus complex as it depends on where the biomaterial will be used and as opposed to blood compatibility, for example [5], the sequence of events involved in the ocular response to biomaterials has not been clearly identified. While the various components affecting biocompatibility are being studied, the interactions between these are not yet well defined. To understand the host response to a biomaterial, knowledge of the structure of the tissues and the cells and proteins present are key. In this chapter, the structure and physiology of the eye will be briefly introduced and the ocular response to biomaterials will be discussed in the anterior and posterior segments.

30.2 Anatomy and physiology of the eye in relation to biomaterial applications

The eye is a major sensory organ of the human body that enables vision. The eyeball, formally called the globe, is housed within the eye socket, a bony invagination of the skull known formally as the orbit. The walls of the globe are comprised of three layers of tissue with specific functions (Fig. 30.1). The sclera is the protective, collagenous outermost layer of the globe. Although opaque and white, the anterior portion of the sclera—called the cornea—is transparent and enables passage of light to the interior of the globe. The uvea is the vascularized middle layer of the globe, and provides nutrients and gas exchange to the avascular tissues of the globe [6]. The uvea also acts as a selective barrier between the blood and fluids of the eye called the aqueous humor. The anterior portion of the uvea—called the iris—is located behind the cornea and is responsible for dilating or constricting the aperture, or pupil, of the eye [6]. The medial portion of the uvea—called the ciliary body—is a complex muscular structure responsible for changing the shape of the lens (as part of the process of accommodation), as well as producing the aqueous humor [6]. The lens, located behind the iris and surrounded by the ciliary body, focuses light onto the innermost layer of the globe, the retina. The retina is an epithelial layer of the globe that converts stimulation by photons into nerve impulses that are transmitted along the optic nerve into the visual centers of the brain. Typically, the anterior structures of the eye up to and including the lens are referred to as the anterior segment of the eye.

30.2.1 The ocular surface

The cornea is a transparent avascular, nonkeratinized epithelial structure, forming one-sixth of the area of the outer wall of the eye. It represents the optical interface between the eye and external environment and functions as an optical element and protective barrier. Together with the lens, the primary function of the cornea is to refract light to focus an image on the retina; therefore, they must maintain their transparency, optical physiology, and structure. The normal human cornea is 500 μm thick and consists of five layers: corneal epithelium, Bowman's layer, stroma, Descemet's membrane, and the corneal endothelial monolayer [8]. The corneal epithelium is a stratified structure, 50 μm thick, consisting

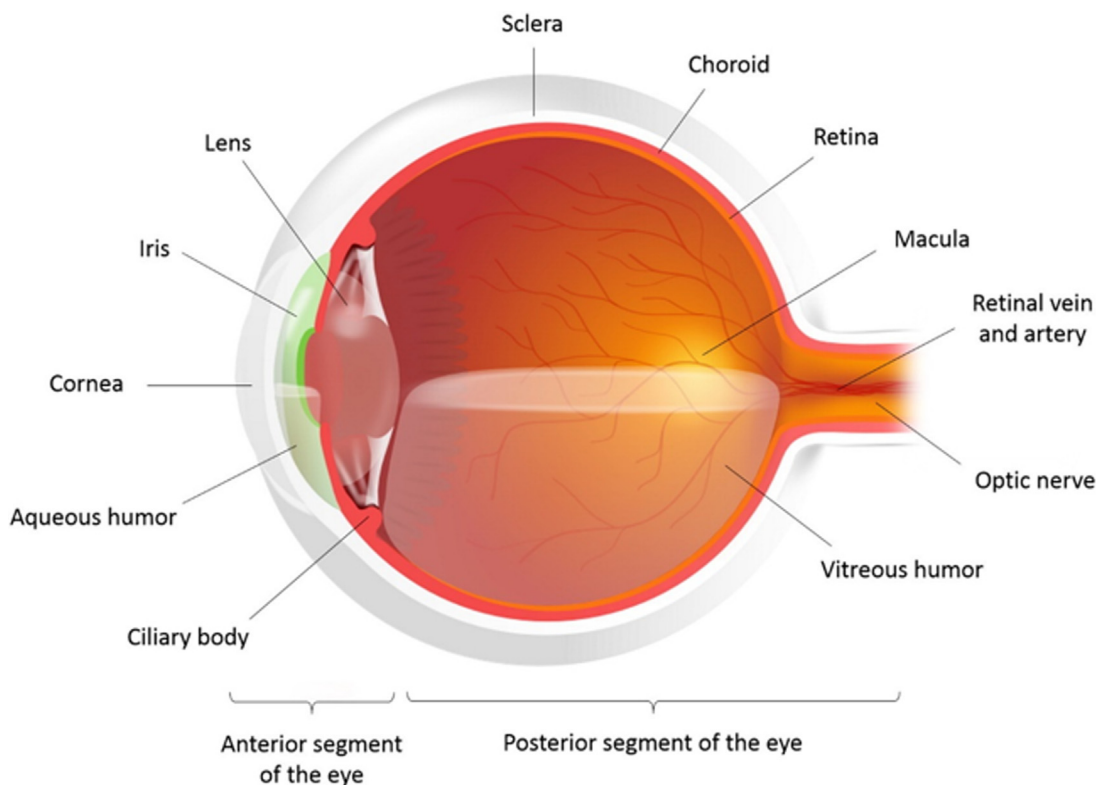


FIGURE 30.1 Anatomy of the eye. Source: Reprinted from Delplace V, Payne S, Shoichet M. Delivery strategies for treatment of age-related ocular diseases: from a biological understanding to biomaterial solutions. *J Control Release* 2015;219:652–68 with permission from Elsevier [7].

of a single layer of squamous superficial epithelial cells, several layers of intermediate wing cells, and a single layer of columnar basal epithelial cells [8] (Fig. 30.2). Superficial corneal cells provide a substrate for the precorneal tear film, which acts as the primary refracting surface of the eye [8].

The cornea is well protected from pathogens and the external environment by tight junctions and the constant self-renewal, lacrimation and blinking, antimicrobial enzymes in tears, and nearby antigens, cytokines, inflammatory mediators or leukocytes that enter the cornea via limbic and/or ciliary body vessels [8]. The population of epithelial cells is maintained by the balance between cell divisions at the limbus and basal layers and cell loss or sloughing at the surface (with an epithelial cell turnover rate of approximately 7 days) [8]. The presence of tight junctions in the corneal epithelial layer plays a vital role in the barrier function of the cornea, protecting intraocular structures against diffusion of substances from the tears, transport of ionic or polar molecules, microbial infections, and other environmental stresses [9–11]. As opposed to the corneal epithelium, the corneal endothelium has limited capacity for regeneration. This monolayer of cells plays a key function in regulating water to maintain hydration of the cornea and allows diffusion of

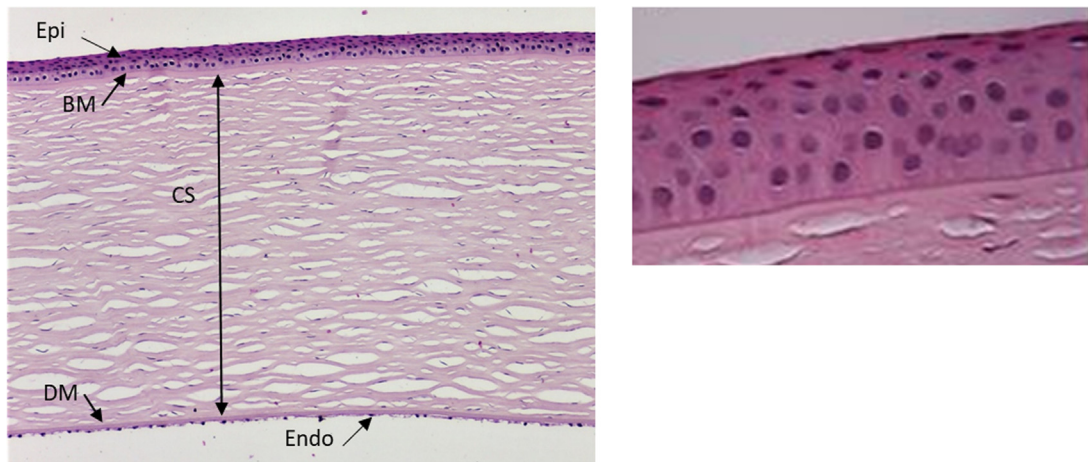


FIGURE 30.2 Histological section of human cornea with the various layers: corneal Epi, BM, CS, DM, and corneal Endo. The stratified structure of the corneal epithelium with a difference in shape between the superficial and basal epithelial cells can be more clearly identified in the picture on the right. *BM*, Bowman's membrane; *CS*, corneal stroma; *DM*, Descemet's membrane; *Endo*, endothelium; *Epi*, epithelium. Source: Pictures courtesy of Denise Hileeto (School of Optometry and Vision Science, University of Waterloo).

nutrients. Cells of the ocular surface and endothelium express integrins (cell membrane receptors), which play a role in cell adhesion, migration, and maintenance of tissue integrity and which expression can be affected by biomaterials [9,12,13]. Corneal and conjunctival epithelial cells, stromal cells, and resident antigen-presenting cells also express toll-like receptors (specifically TLR2, 3, 4, 5, and 7), which are involved in inflammatory and immune cell activation and recruitment [14].

The conjunctiva covers the surface of the eye and lines the undersurface of the eyelids. The conjunctival epithelium has a stratified structure and similarly to the corneal epithelium, its cells express various integrins and receptors and tight junctions (in the superficial layers). Goblet cells, secreting mucus, are also present in the conjunctiva.

The tear film is responsible for the hydration, lubrication, and nutrition of the ocular surface while providing a barrier to pathogens and particulates. The tear film is viewed as layers consisting of the outermost nonpolar lipid layer, the polar lipid layer, the aqueous–mucin layer, and a glycocalyx layer covering the cornea (Fig. 30.3). Components of the tear film are secreted by different epithelial and glandular tissues, such as the meibomian glands (the lipid layer), lacrimal glands (the aqueous layer), and the goblet cells within the conjunctiva (the mucin layer) [15]. Mucins are highly glycosylated proteins that are both secreted and membrane-associated.

Over 1500 tear proteins have been identified in tears from healthy subjects, with lysozyme, lactoferrin, tear lipocalin, and secretory immunoglobulin A (sIgA) being the main proteins found in the tear fluid [18]. Tears contain cytokines (such as IL-1 β , IL-6, IL-8, and IL-12), chemokines (such as CCL2, CXCL1, and CXCL8) and growth factors, which are produced by resident cells of the ocular surface and infiltrating immune cells and play an essential role in corneal homeostasis and inflammatory processes [18–20]. It is important

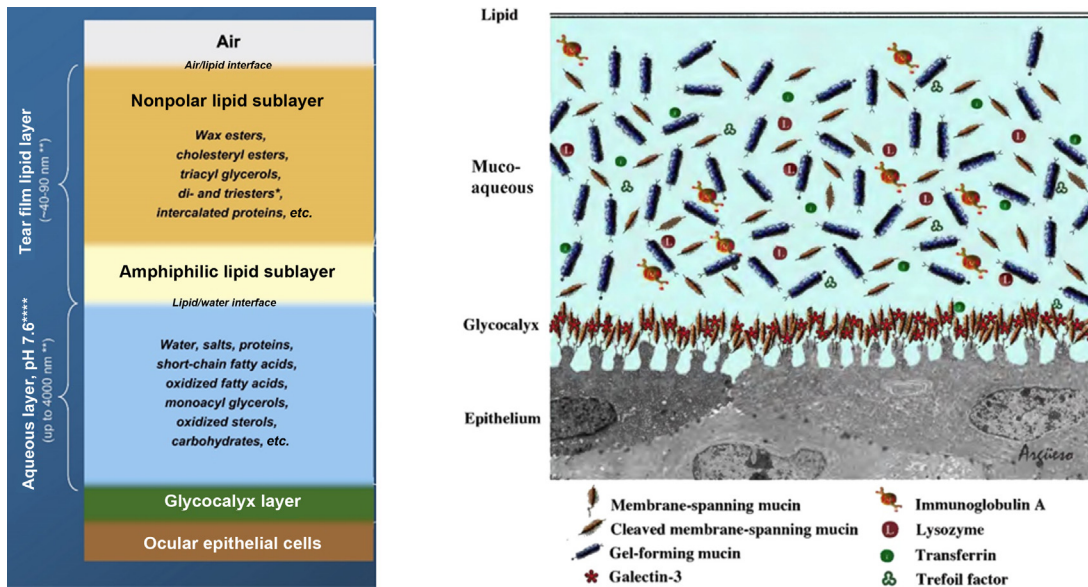


FIGURE 30.3 The tear film structure. The left scheme presents the overall structure while the right scheme presents a more detailed view of the mucins and galectin of the glycocalyx, soluble mucins, and proteins in the muco-aqueous layer. Source: Reprinted from Butovich IA. *The meibomian puzzle: combining pieces together*. *Prog Retin Eye Res* 2009;28(6):483–98; Willcox MDP, Argüeso P, Georgiev GA, et al. *TFOS DEWS II tear film report*. *Ocul Surf* 2017;15(3):366–403 with permission from Elsevier [16,17].

to note that the composition of the open-eye and closed-eye tears are significantly different from each other. Closed-eye tears contain higher levels of albumin, fibronectin, complement proteins, sIgA, and plasmin compared to open-eye tears, with sIgA representing up to 80% of the total protein content [21,22]. Furthermore, a large number of neutrophils have been observed in closed-eye tears [23,24]. These differences have the potential to impact the ocular response to biomaterials.

With its lack of blood vessels and inherent protection mechanisms, the cornea is considered an immune privilege tissue, whereby to prevent vision loss, minimal inflammation, and immune response may occur [25,26]. However, the presence of a biomaterial may affect this protected environment and trigger an inflammatory response that can damage the ocular surface [24,27,28].

30.2.2 The anterior segment of the eye

Within the anterior segment of the eye are two fluid-filled compartments: the anterior chamber, located between the corneal endothelium and the iris, and the comparatively smaller posterior chamber, located between the iris and the lens, and bounded along its equator by the ciliary body [29]. The ciliary epithelium and the endothelium of capillaries within the ciliary body form a physical and immunological barrier called the blood-aqueous barrier (BAB). The BAB, along with other similar barriers throughout the eye,

confer the properties of immune privilege to the intraocular environment [30,31]. Immune privilege is believed to be an evolutionarily conserved trait that protects critical tissues from inflammation due to injury or infection [31,32]. Located behind the iris, the lens is bathed in aqueous humor and connected along its equator to the ciliary body by suspensory ligaments known as the Zonule of Zinn. Physiologically, the lens is comprised of three parts: (1) the lens capsule, a type-IV collagen-rich basement membrane surrounding the lens; (2) the lens epithelium, a monolayer of lens epithelial cells on the inner surface of the anterior lens capsule; and (3) the lens fibers, transparent, enucleated cells filling the volume of the lens [29].

Both compartments are filled with the aqueous humor, which provides nutrients and removes metabolic byproducts from the avascularized tissues of the cornea and lens [29]. Although often compared to a blood plasma dilution, the aqueous humor has a unique chemical composition. By concentration, the aqueous humor is almost entirely water, with 500 times less protein than plasma (11 mg/100 mL compared to 6 g/100 mL in plasma) [33,34]. Immunoglobulins, or antibodies are found at less than 1% of their serum concentration, suggesting that other mediators may be responsible for the intraocular immunological response [35,36].

30.2.3 The posterior segment

The posterior segment contains a gel-like substance called the vitreous humor within the hyaloid membrane and is also formed by the retina, the choroid, and the sclera; these three tissue layers adhere to each other under normal conditions (and are sometimes referred to as the posterior wall). The choroid is a membrane rich in blood vessels, which becomes the ciliary body in the anterior segment, while the sclera is the tough fibrous outer layer protecting and shaping the eyeball, which is significantly modified in structure to form the cornea anteriorly. The inner layer of the posterior segment, the retina, consists of two layers: the pigmented layer and the neural layer (Fig. 30.4). The pigmented layer is a monolayer of retinal pigment epithelial (RPE) cells which absorb light and prevent light scattering in the eye but also contribute to the blood–retina barrier (through RPE cells' tight junctions) and act as phagocytes of light-damaged photoreceptors. Photoreceptors (rods and cones), bipolar cells, and ganglion cells form the neural layer of the retina and are involved in processing responses to light. In the back of the eye, an opening allows the optic nerve to leave the eye and also houses the central artery and vein of the retina; this area is referred to as the optic disc as well as the blind spot as no photoreceptors are present. Glial cells, which include Müller cells and astrocytes, are the immune cells residing in the retina and optic nerve. They play a key role in homeostasis and protection of the retinal neurons. Pathological conditions and injuries to the retina, such as increase in intraocular pressure (IOP), ischemic damage, or neuroinflammation, result in the activation of glial cells (a process called gliosis), a response which can also be induced by the presence of a biomaterial. This process can have both cytoprotective and cytotoxic effects on the retina [37].

As opposed to the aqueous humor which forms and drains continuously, the vitreous humor is a gel composed mainly of water (98%), collagen type II fibrils, and the glycosaminoglycan hyaluronic acid. The ocular environment is also rich in proteins and enzymes

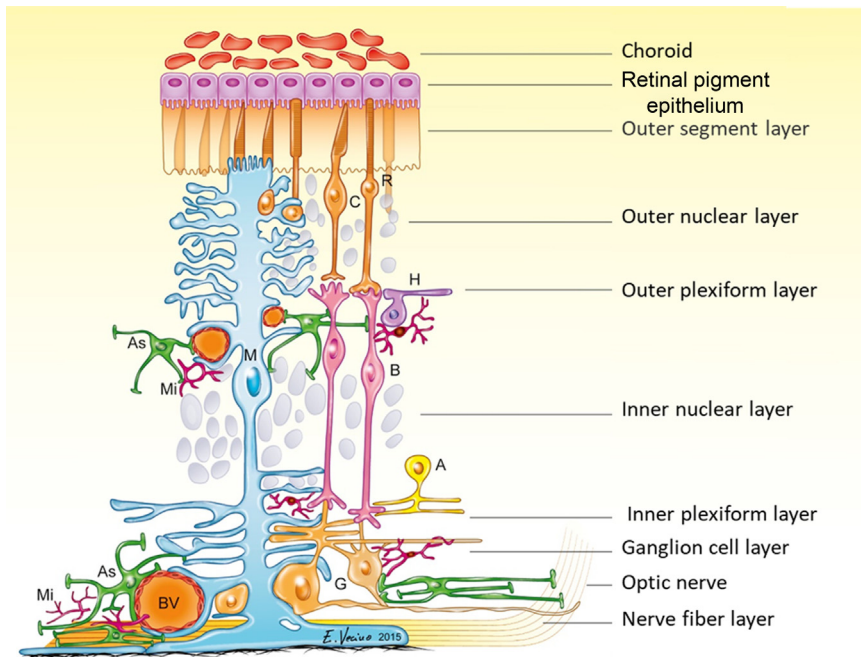


FIGURE 30.4 Anatomical structure of the retina. The different layers of the retina as well as cell types are depicted: amacrine cells (A), astrocytes in green (AS), bipolar cells (B), cones (C), ganglion cells (G), horizontal cells (H), Müller cells in blue (M), microglia in red (Mi), rods (R), cones (C). Note the interaction of cells with the BV. BV, Blood vessels. Source: Reprinted from Vecino E, Rodríguez FD, Ruzafa N, Pereiro X, Sharma SC. Glia-neuron interactions in the mammalian retina. *Prog Retin Eye Res* 2016;51:1–40 with permission from Elsevier [38].

such as metalloproteinases (MMPs). Aging and ocular diseases affect the posterior segment by creating a subinflammatory state, which has strong biocompatibility implications in the design of biomaterials that are aimed at treating ocular disease and preserving vision. For example, with macular degeneration, changes in protein concentrations as well as increases in complement activation products and MMPs have been observed in the vitreous and retinal space; such conditions have the potential to impact the stability of the biomaterial and the material-induced ocular response [39–41].

30.3 Ocular response to biomaterials in the anterior chamber

30.3.1 Ocular response to contact lens and artificial cornea materials

Contact lenses (CLs) represent the most widely used medical device (with an estimated 125 million wearers worldwide) and the most common biomaterial to interact with the ocular surface [42]. Soft CLs are hydrogel polymers typically made either from poly(2-hydroxyethylmethacrylate) or silicone hydrogels [43]. In order to increase oxygen transmissibility of these materials to reduce the risk of corneal hypoxia, all of these polymers

are surface treated with monomers and wetting agents added to their surface [43]. Rigid gas permeable (RGP) CLs used in orthokeratology or with keratoconus patients were originally manufactured with polymethyl methacrylate (PMMA), which has a low oxygen permeability [44]. To increase oxygen permeability and improve biocompatibility, RGP lenses now use, most commonly, silicone or fluorosilicone acrylate materials [44].

While millions of lens wearers benefit from improved vision with comfort and limited complications, issues of biocompatibility exist with lens wearers being at increased risk of corneal infiltrates and microbial keratitis, significantly more so with overnight lens wearers [45–47]. As CLs are being increasingly considered for drug delivery system, monitoring, and diagnostic devices [48,49], it is important to gain a better understanding of biomaterial interactions with the ocular surface.

Classically, the first consideration for biomaterial interaction in the body is protein adsorption, with the two typical proteins considered being albumin and fibrinogen, as most implants will interact with blood. However, the ocular surface is a unique system where CLs will interact with over 1500 proteins present in the tear film with concentration significantly different from blood plasma [18]. Protein adsorption on CL will affect bacteria adhesion, visual acuity, and the cornea response. Protein adsorption depends on the lens material and surface charge, as well as the concentration, charge, and structure of the protein and similarly to blood-material interactions is also a competitive process [50–53]. An important consideration with CLs is also conformational change as protein denaturation once adsorbed on the lenses may also trigger a physiological response from the cornea [54,55]. Lysosyme, a tear protein with antibacterial and antiinflammatory functions, has been shown to adsorb differentially on CL materials both from a concentration and denaturation perspective, with the latter leading to a loss of functionality and an inflammatory response in corneal epithelial cells [56,57]. Lipids are also an important component of the tear film and have been shown to adsorb on CLs [58]. The adsorption of lipids and proteins affects the tribological behavior of CLs, which can lead to higher frictional forces during blinking and impact biocompatibility with the cornea and the eyelids [59]. Protein adsorption on CLs and its physiological impacts are not yet fully understood [50]. The development of more sensitive technologies and physiologically relevant *in vitro* and *in vivo* assays and models have the potential to contribute significantly new knowledge to how protein adsorption affects lens biocompatibility [50,60,61].

CLs interact with the cornea and eyelids and more specifically with corneal and conjunctival cells (note that the cornea is highly innervated, and while contact lens wear has been shown to affect neurons in the cornea, limited research currently exists on the mechanisms related to neural biocompatibility). Oxygen and ionic permeabilities are important material properties to maintain cornea health and homeostasis. *In vivo*, ocular surface cell response to CL materials can be assessed visually using microscopy (slit lamp biomicroscope, confocal microscope) or optical coherence tomography. These techniques provide information on the ocular response to biomaterials by characterizing changes in layer thickness, number of cells, and cell size in the corneal epithelium as well as the corneal endothelium. Instillation of sodium fluorescein, a fluorescent dye, on the ocular surface allows to assess the staining pattern under blue light to determine if erosion or cell loss has been induced by the biomaterials. While fluorescein has been used for decades to assess corneal health, note that it is not yet clear what fluorescein stains and why some

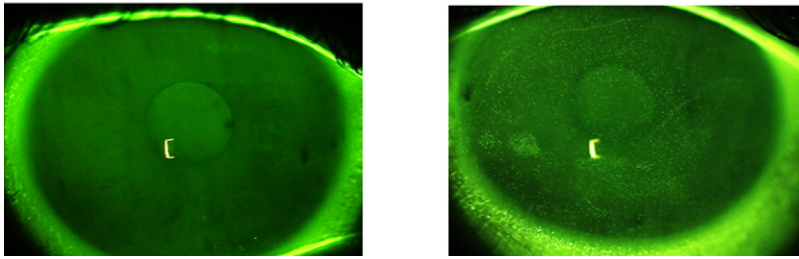


FIGURE 30.5 Two eyes stained with sodium fluorescein. The eye on the left shows no corneal staining, whereas the eye on the right shows classic micropunctate corneal staining. Source: *Pictures courtesy of the Centre for Ocular Research and Education (CORE, University of Waterloo).*

cells uptake fluorescein but not others especially in the context of CL and lens cleaning solutions [62–64] (Fig. 30.5). While it may be difficult to characterize ocular cell response *in vivo*, several assays are now available to measure cytokine concentration in the tear film, allowing to assess if the presence of a biomaterial on the ocular surface induces an inflammatory or immune response [65–67]. In animal experiments, the ocular irritation using the Draize scoring system can also be used and histopathology allows to determine the presence of an inflammatory response, scarring, or toxicity. The expression of integrins (see Section 2.1) can also be characterized by immunohistochemistry to provide evidence that the presence of a biomaterial does not alter tissue integrity and functionality. *In vitro*, the ocular response to CL materials has often focused on assessing material toxicity on corneal epithelial cells, either as a monolayer or as a stratified culture, thus providing a limited understanding on the potential mechanisms for biocompatibility [62]. Recent *in vitro* studies have been investigating changes in integrin expression, the presence of tight junctions, synthesis of cytokines and metalloproteinases, as well as production of reactive oxygen species, allowing to better assess ocular response to drug delivery and bandage CLs and/or ocular surface materials in general [68,69]. The high drop-out rates in CL wearers has also led some researchers to question if ocular inflammation and biocompatibility may play a role in discomfort [70].

To repair corneal tissue defect, collagen- and gelatin-based biomaterials are being investigated. While for CL materials, protein adsorption should be avoided, in the case of artificial cornea, corneal patches, or ocular adhesives, the presence of protein is important to promote cell adhesion, migration, and proliferation to support the production of an epithelium and/or endothelium and to contribute to wound closure. Cell-binding peptides, tethering growth factors, and nanotopography have also been shown to improve cell adhesion [68,71–73]. Consideration also needs to be given to how protein adsorption, cell adhesion, and extracellular matrix (ECM) formation will occur on the biomaterial to prevent light interference.

30.3.2 Ocular response to intraocular lens

Foldable IOLs have become common practice in cataract surgery as they reduce incision size and damage to corneal and uveal layers of the eye. The hydrogels and surface chemistries commonly used in IOL manufacturing include: (1) hydrophilic acrylic made of poly

(2-hydroxyethyl methacrylate) (HEMA), (2) hydrophobic acrylic based on PMMA, and (3) hydrophobic silicone hydrogels. Copolymers, such as hydrophobic IOLs made of acrylate-methacrylate copolymer, are being developed to address biocompatibility issues and improve/protect vision (addition of UV blocker, optical clarity) [74]. With IOLs, uveal (anterior chamber response and inflammatory cell deposition) and capsular (the response of residual lens epithelial cells) biocompatibility are considered separately; IOLs present good uveal biocompatibility and complications tend to be due to posterior capsular opacification (PCO), although recent results suggest that macrophage activation could potentially contribute to PCO [75–77].

PCO is one form of secondary cataract and the most frequent complication of cataract surgery, occurring in 38.5% of patients after 3 years, despite decades of efforts to improve surgical techniques and IOL design and surface modifications [78,79]. In PCO, some surviving lens epithelial cells undergo transdifferentiation into myofibroblast cells via a process called the epithelial-mesenchymal transition, leading to the pathological expression of the contractile protein α -smooth muscle actin, increased expression of the mobility protein vimentin, and a loss of expression of type-IV collagen and the intercellular cadherin E-cadherin [80]. These myofibroblasts induce fibrosis by overproducing proteins of the ECM such as fibronectin, type-I and type-II collagen, and tenascin [80]. To improve IOL biocompatibility, approaches have focused on preventing lens epithelial cells (LEC) adhesion to the biomaterial with lens design (square vs round edges) and biomaterial modifications to impart different properties between the front and back of the IOL (hydrophobic front, hydrophilic back) [81,82]. Functional modifications to prevent protein adsorption (such as fibronectin) and cell adhesion have been developed and shown success in vitro and in vivo with polyethylene glycol (PEG) [83], *N*-vinyl pyrrolidone [84], hirudin [85,86], and heparin [87] coatings.

Damage to the BAB during phacoemulsification (phacoemulsification is the process used to emulsify the lens prior to replacing it with an IOL) may permit blood interaction with IOLs, although blood components entering the anterior and posterior chambers will likely be diluted by aqueous humor. However, despite the potential for interactions of IOL materials with blood proteins and inflammatory cells, few studies have examined these [88,89]. IOLs are also implanted in patients with uveitis (an inflammation of the uvea) or diabetes, where the BAB is significantly compromised and IOLs may thus be exposed to a subinflammatory state (increase in inflammatory cells, cytokines, and proteins) compared to healthy eyes, which will impact biocompatibility [90–92]. Macrophages play a crucial role in the wound-healing response to biomaterials and have been observed on the surface of IOLs explanted from human patients and suggest that differences exist between biomaterials (Fig. 30.6) [93–96]. Despite mounting evidence of macrophage presence in PCO, the leukocyte response to IOL materials (often referred to as uveal biocompatibility) and its potential contribution to the development of PCO have not been well investigated, and thus the role of material chemistry and other design factors remain poorly understood. In an animal model of PCO, depletion of macrophages reduced the number of lens epithelial cells in the center of the posterior capsule, [97] suggesting that macrophages may play a role in lens epithelial cell mobility during PCO. Macrophage interaction with IOL materials was also shown to induce an inflammatory phenotype disruption in lens epithelial cells in vitro [98] while a recent study also reported a positive correlation between the level of

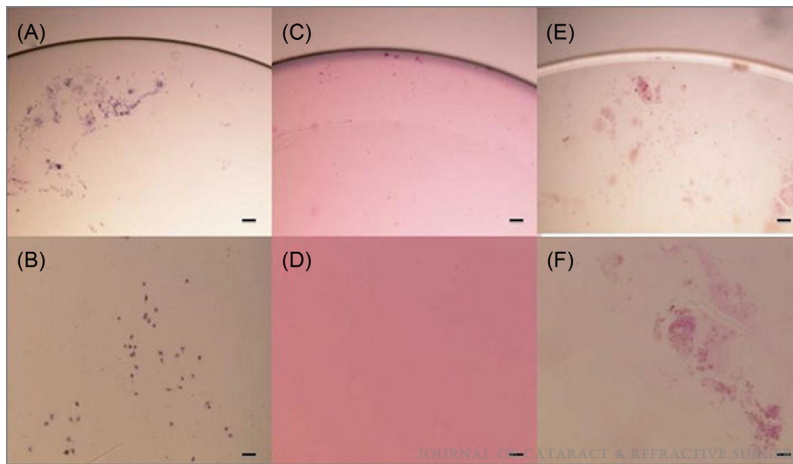


FIGURE 30.6 Histology of IOLs explanted more than 24 months after surgery. (A and B) Poly(methyl methacrylate) IOL. (C and D) Silicone IOL. (E and F) Acrylic IOL (A, C, and E = original magnification $\times 40$; B, D, and F = original magnification $\times 100$) (H&E stain; bar = 100 μm). IOLs, Intraocular lenses. Source: Reproduced from Ishikawa N, Miyamoto T, Okada Y, Saika S. Cell adhesion on explanted intraocular lenses: Part 1: Analysis of explanted IOLs. *J Cataract Refract Surg* 2011;37(7):1333–8. ©2011 with permission from Lippincott Williams.

macrophage-derived chemokine and PCO in children [99]. The recent studies on macrophage response to IOL material suggest that inflammation may play a role in PCO and introduce the potential for new strategies to improve the biocompatibility of IOLs.

30.3.3 Ocular response to glaucoma shunts and (noncontact lens) drug delivery systems in the anterior eye

Other devices placed in the anterior segment of the eye are typically designed to address issues of dry eye, or remediate disease states in the anterior or posterior segments of the eye such as uveitis, glaucoma, or age-related macular degeneration. These devices include punctual plugs, glaucoma drainage implants as well as drug delivery systems such as small rods, films, or microspheres. Control over surface properties is a key parameter for biocompatibility where in several applications, there is a need to facilitate tissue adhesion (to reduce device micromotion) but without promoting fibrosis and maintaining lubricity which is also necessary for success of glaucoma drainage implants. These devices have been typically made of nonsurface-treated hydrophobic polymers such as polyvinyl alcohol (PVA), ethylene vinyl acetate, and silicones (aqueous drainage shunts, punctual plugs) but polypropylene (PP) and PMMA are also being used for the plate in drainage devices [100]. Intrasccleral implants commercially available can either be absorbable (collagen or crosslinked hyaluronate), biodegradable [polylactic acid (PLA), polyglycolic acid, or copolymer polylactic-*co*-glycolic acid (PLGA)], or nonabsorbable such as the acrylic implant (T-flux) [101,102]. The complications and failure rates (up to 50% in some cases) due to filtration rate and fibrosis in drainage devices remain a serious concern [103,104] and

place these devices as the secondary choice, especially in young patients and older adults [105]. Protein adsorption and cell adhesion are recognized to be significant contributors to fibrotic response to biomaterials. The increase levels of cytokine TGF- β , which follow surgery and may persist due to chronic inflammation in the tissue, can also contribute to the persistent differentiation of fibroblasts into myofibroblasts and ensuing fibrosis [106]. Several approaches, such as using naturally-derived biomaterials (glutaraldehyde-crosslinked gelatin [107]), new biomaterials (e.g., hydrophobic terpolymer, polytetrafluoroethylene (PTFE)-polyvinylidene (PVD)-PP or SIBS [poly(styrene-block-isobutylene-block-styrene [108]))], and antifibrotic drug delivery drainage implants (cyclosporine or diclofenac sodium) have been successful in clinical trials and animal models. Furthermore, new designs for glaucoma drainages, such as the trabecular microbypass stent, iStent, an heparin-coated, nonferromagnetic titanium device, have shown very promising results with lower complications of tissue encapsulation [102,109]. Different fibroblast subpopulations have also been identified in the anterior eye, whereby some fibroblasts can synthesize significantly more matrix proteins and thus contribute to tissue fibrosis [110]. This knowledge has recently been applied to successfully develop a novel biodegradable microstent and drug delivery system to reduce fibrosis and scarring (around the surgical incision) [111].

30.4 Ocular response to biomaterials in the posterior segment

One of the most common development of biomaterials for the posterior segment is toward intravitreal therapeutic delivery systems for disease such as age-related macular degeneration, uveitis, or diabetic macular edema. Biomaterials have included natural and synthetic polymers and hydrogels such as silicone, chitosan, cellulose, PVA, and PLGA. Micelles, microparticles, nanoparticles, crosslinked hydrogels and rods are being explored or already used clinically (for a thorough review of ocular hydrogel materials, see Delplace et al. [7]). Thermo-responsive hydrogels such as poly(*N*-isopropyl acrylamide) are also being designed to encapsulate and release therapeutic agents (i.e., bevacizumab and ranibizumab) or retinal stem-progenitor cells [112–114]. Controlled degradability is an important component of ocular response for intravitreal biomaterials as surgical removal increases the risks of ocular complications. Both upon implantation and degradation, the biomaterial needs to induce minimal change in the vitreous volume as this will increase IOP and is thus an important determinant for biocompatibility. The response of retinal cells to both the polymer but also to the degradation products needs to be characterized. Toxicity, cytoskeletal organization, and inflammatory cytokine release have been most commonly evaluated *in vitro* with microglia/macrophages and RPE cell lines [115]. Intraocular biocompatibility is then assessed from morphological (optical coherence tomography and histology) and functional (electroretinography) perspectives [116]. Surface charge of liposomes (cationic) has been shown to increase toxicity and inflammation. A recent study comparing the intravitreal inflammatory response induced by microspheres and rods of PLGA also has suggested that size, shape, and/or surface area could significantly affect the ocular response, emphasizing the need for further research in the determinants of biocompatibility of intravitreal implants [117]. Furthermore, although the biomaterial itself may be biocompatible, the drug delivered may lead to complications due to its targeted delivery; increase in IOP or lens clouding (secondary glaucoma, and

cataract) have been reported with corticosteroids [118]. Thus while intravitreal drug delivery systems have significant advantages over repeated injections, the pharmacodynamics of this localized, long-term delivery of the therapeutic agent needs to be further examined to support the development of biocompatible ocular delivery systems. Physiological differences between animals and humans can limit translation of results and animal models need to be carefully considered when evaluating the ocular response of intravitreal drug delivery devices [113].

Ocular complications, such as secondary cataract, gliosis, and retinal inflammation, of current vitreous substitutes (silicone oil, perfluoro-*n*-octane, perfluorohexyloctane) have led to an interest in developing new hydrogel biomaterials. While hydrogels synthesized from natural polymers such as chitosan, hyaluronic acid, alginate, or collagen have good biocompatible properties from a cell/tissue perspective for the posterior segment, degradation, and poor rheological properties, despite the use of various crosslinking strategies, make them poor candidates for this use (for a complete review of hydrogels, refer to Su et al. [119]). Synthetic hydrogels (PVA, PHEMA), PEG-based hydrogels, and smart hydrogels (such as responding to temperature change or ionic strength) are being developed [120]. Biocompatibility with retinal tissues is paramount and as discussed above, ocular response to degradation products also needs to be considered. In addition to biocompatibility toward retinal pigment cells, the vitreous substitutes should not activate glial cells. Increase in glial fibrillary acidic protein in glial cells as well as the presence of galectin-3 (a marker present during neuroinflammation) have been observed with some vitreous substitutes both in *in vitro* and *in vivo* models [121,122]. However, there is yet limited explanation to the reason of their lack of biocompatibility (is it due to their chemistry, degradation product, etc.). Increase in phagocytic and antigen-presenting cells in the retina has also been associated with some vitreous substitutes, suggesting a material-induced immune response.

Biomaterials are also being designed as scaffold and films for cell delivery of RPE cells and photoreceptors. While the nanofiber structure of the biomaterial is a key determinant in cell survival and organization, the difference in mechanical properties compared to the native tissue can lead to tissue damage and implant fibrosis [123,124]. As observed in other tissues, such as in bone, the acidic degradation products of PLA/PLGA scaffolds are also believed to induce a chronic inflammatory response in the retinal space [123,125].

Retinal implants are new technologies used to restore vision using implanted electrodes to stimulate retinal neuron cells. Implants use silicon-based electronic devices that are embedded in a soft or hard shell, which needs to be biocompatible for long-term implantation (for a detailed review on the technologies being developed, see Zeng et al. [126]). The electrodes/implants can be placed either on top of the retina, between the retina and the choroid, or on the posterior surface of the sclera. Each location has specific requirements around the ocular response to the “shell” as it will directly interact with different tissues and physiological fluids. Ocular cell biocompatibility will be affected by the physicochemical properties of the biomaterial, which may also change over time due to long-term implantation. Responses, such as fibrous encapsulation and hypotony, are complications similar to what has been observed with glaucoma drainage devices (Section 3.3) and appear to be dependent both on surgical techniques and biomaterials [127]. The retinal neural cells/electrode interface is common to all implants and can be a challenging problem to address, with potential for cell

cytotoxicity and protein adsorption on the electrodes that will affect the long term efficacy of the stimulation [128]. Number, shape, and materials used for the electrodes have all been shown to play a role in ocular response [126]. Morphological retinal changes were recently reported with retinal prosthesis implants [129].

30.5 Conclusion

Ophthalmic biomaterials include biopolymers, biodegradable synthetic polymers, and synthetic polymers. Several surface modifications, such as coatings or patterning, and drug delivery approaches have been designed to improve biocompatibility in the anterior and posterior segments of the eye. While many new devices and biomaterials have been successfully developed, the exact mechanisms involved in the tissue reaction to the device and its biocompatibility are still poorly understood due to the complex interactions between the various components of the ocular system. The role of proteins and cells has been recognized to play a role in biocompatibility but needs to be further investigated at the molecular level to clearly identify the pathways involved and provide more specific targets to improve long-term biocompatibility. As many biomaterials and ophthalmic medical devices are being developed to treat ocular diseases, the inflammatory conditions and changes to the ocular environment associated with these diseases need to be carefully considered in the design and testing phase to ensure biocompatibility in a subinflammatory environment.

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Skin responses to biomaterials

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31.1 Introduction

The skin is a natural physiological barrier organ that separates the body from the outside environment. It can regulate the immune cells and the body's temperature, and produce and activate several bioactive substances to maintain physiological balance. It also prevents bacterial infection and identifies potential threats [1]. The skin maintains its homeostasis under physiological conditions, and its balance is disturbed when attacked by an external environment, such as burns, instrument damage, ulcers, infections. Skin damage has also become a common medical problem around the world, with about 300 million patients suffering from acute injuries globally every year. The prevalence of chronic wounds has increased rapidly in recent years [2]. The chronic wounds are heterogeneous in their presentation and etiology. Notably, there is high protease activity, large-scale infection and biofilm formation, ischemia or hypoxia in the tissue, recurrent injury due to neuropathy, or cellular failure leading to gangrene associated with chronic wounds. Chronic wounds most often occur as venous leg ulcers, pressure ulcers, or foot ulcers and are found at elevated rates in patients with diabetes and obesity. The body will not be able to heal fully on its own [3,4].

There are four distinct stages in normal wound healing process, including hemostasis, inflammation, proliferation, and remodeling, and each stage corresponds to the time scale (see Fig. 31.1). To understand the healing process better, we need to explore the mechanisms of wound healing. Healing involves two different mechanisms: regeneration or repair [5]. A series of events following skin injury has been extensively studied, involving multiple cell types and signaling to induce wound healing. These stimuli prompt progenitor cells to reach the site of the damaged tissue. However, this process is internal and highly coordinated during development, and loses its potency in adults [6]. This leads to a disturbance

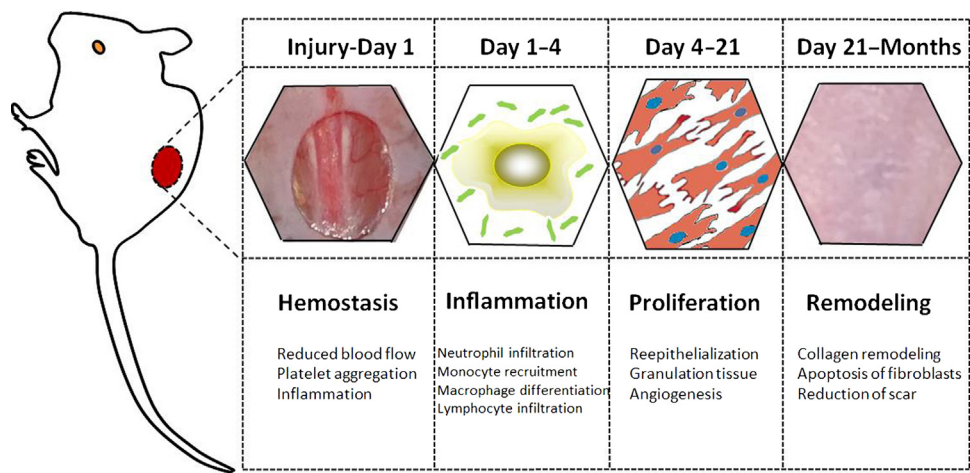


FIGURE 31.1 Wound healing phases. Scheme representing the four different phases of normal wound healing process, including hemostasis, inflammation, proliferation, and remodeling.

of the extracellular matrix (ECM), commonly referred to as scarring. The mechanisms underlying adult skin tissue healing as scarring are unclear [7].

Biomaterials can be used as platforms to generate stimuli that promote skin regeneration. Biomaterials are a powerful tool that modify the host microenvironment due to their properties obtained through the fabrication process. Surface chemistry, topography, mechanical properties, and degradation products combined with biological signals can promote an efficient regeneration. They are multistimuli, not limited to biochemical signals. They can induce cell recruitment and activate a highly controlled self-secretion of growth factors (GFs) and cytokines that stimulate the production and organization of the ECM.

The biocompatibility between biomaterials and organisms has always been the theme in research. Biocompatibility refers to the ability of living tissue to react to inactivated materials. It generally refers to the compatibility between the material and host, including histocompatibility and blood compatibility. Biomaterials are foreign bodies to the skin, which produce some immune response or rejection in the body. If biomaterials are to be successful, the host must be responsive, at least without harmful effects.

In this article, we give a general description of the skin tissue. More importantly, we investigate the skin response to biomaterials through inflammatory responses, hypersensitivity responses, and stimulation responses. The stimuli responses are explored from biomaterial physical properties, biomaterial bioactive strategies, biomaterial mechanical properties, and stimulus signal as well as metal ions and inorganic compounds. Finally, we summarize the role of biomaterials in skin tissue engineering. We expect to find better treatments for skin problems.

31.2 General description of the skin tissue

The skin is a natural physiological barrier organ that separates the body from the external environment. It has many physiological functions such as secretion, feeling, avoiding

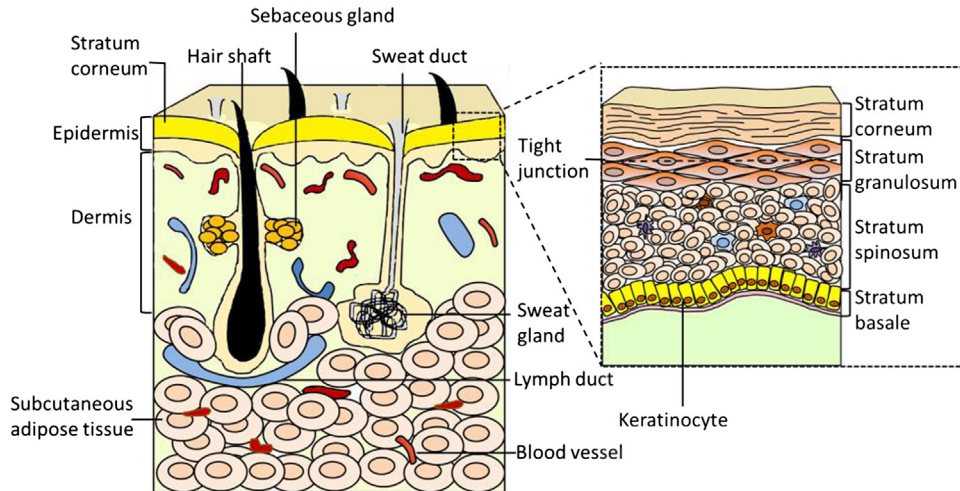


FIGURE 31.2 Structures of the skin tissue. Skin consists of three main parts: the epidermis, dermis, and subcutaneous tissues, alongside with other important components such as the vascular system and skin appendages. The epidermis is divided into four layers including the stratum basale, spinosum, granulosum, and corneum.

dehydration, and participating in the regulation of the immune cells and the body's temperature [1]. Furthermore, skin also produces and activates several bioactive substances such as hormones, serotonin, vitamin D, neuropeptides, catecholamine, and cytokines to maintain physiological balance [8–10]. In addition, it also includes different sensors to prevent bacterial infection and identify potential threats [8,11].

Anatomically, the skin consists of three main parts: the epidermis, dermis, and subcutaneous tissues; and contains the unique cells, ECM, and other skin appendages such as sweat, sebaceous glands, hair follicles, and nails as well as mechanic and temperature sensors [12,13] (see Fig. 31.2). Different cell types including immune cells and nonimmune cells in the skin induce various immune responses to external stimuli, and these immune cells are conducive to understand the cutaneous immune response as well as the mechanism of disease occurrence [12].

The epidermis is the outermost skin that forms the chemical and physical barrier between the internal body and the external environment. It consists of different cell populations, including keratinocytes, Langerhans cells, Merkel cells, and melanocytes. So far, the number of keratinocytes is the most abundant in the epidermis [11]. Structurally the epidermis is divided into four layers including the stratum basale, spinosum, granulosum, and corneum [14]. The stratum corneum is the outermost layer of the epidermis. It not only can prevent outside water molecules and alien organisms from entering the body through the skin, but also prevents water loss in the body. Stratum basale is the deepest layer of the epidermis. It is also the boundary between the dermis and the epidermis. It is composed of stem cells called basal cells which can divide to form keratinocytes to begin migrating superficially [14,15].

The dermis is located between the epidermis and subcutaneous tissue. It contains collagen, blood vessels, nerves, elastin, and sweat glands. and controls the regional variation in skin thickness. Collagen are the primary proteins found throughout the dermis. Proper

collagen deposition and remodeling could improve the skin strength and lead to better healing outcomes [16]. Collagen is produced by fibroblasts, the most abundant cell type in the dermis. Fibroblasts not only produce collagen, but also elastin and other proteins such as proteoglycans. It plays an important role in the remodeling of the skin tissue and wound healing processes. In addition, fibroblasts secrete various GFs such as the cytokines, the transforming GF beta, and the matrix metalloproteinases (MMPs) to affect keratinocyte proliferation and differentiation [17].

The subcutaneous tissues are located between the dermis and muscle. It can keep warm and protect deep tissues from wounds. It contains the subcutaneous fat, blood vessels, superficial fascia, and nerves. The subcutaneous adipose tissue is the most abundant and distributes throughout the body. It can serve energy, pad, and adjust the temperature. It contains a variety of immune cells such as macrophages, B cells and T cells, and plays an important role in the skin immune response. It is also essential for skin defense against foreign bacterial infections. The skin appendages of the skin include sweat glands, sebaceous glands, hair follicles, and nails which regulate the skin [1,12,18]. Sweat is essential for temperature regulation [14]. Sebaceous glands lubricate and waterproof the skin and hair [19]. Hair follicles contribute to maintain body temperature and perceive touch sensation.

31.3 Skin responses to biomaterials

The host has different immune responses to various substances. It is related to the chemical structure, chemical composition, degree of polymerization, additives, and the metal composition of the material. Therefore, the immunological evaluation of biomaterials should be based on the general biocompatibility including tissues and blood. In order to ensure the safe application of biomaterials in the human body, observation and research must be carried out on the molecular level. Biomaterials are foreign bodies to the skin, which will produce some immune response or rejection in the body. If the biomaterial is to be successful, the host must be responsive, at least without harmful effects. Here, we review the reactions of the skin to biomaterials including inflammatory responses, hypersensitivity responses, and stimulation responses. The stimuli responses are explored from biomaterial physical properties, biomaterial bioactive strategies, biomaterial mechanical properties, and stimulus signal as well as metal ions and inorganic compounds.

31.3.1 The inflammatory response induced by biomaterials on skin

Inflammatory response is an important part of the response between biomaterials and host. Long-term implantable biological materials often cause local inflammation of the skin. Macrophages play a central role in skin wound healing and tissue homeostasis. They are also one of the main cell components of the inflammatory response (see Fig. 31.3). When the biomaterial stimulates the skin, macrophages are activated which will release proinflammatory cytokines and adhesion molecules to act on the blood vessel wall, change the permeability of the blood vessel wall, and make white blood cells such as neutrophils

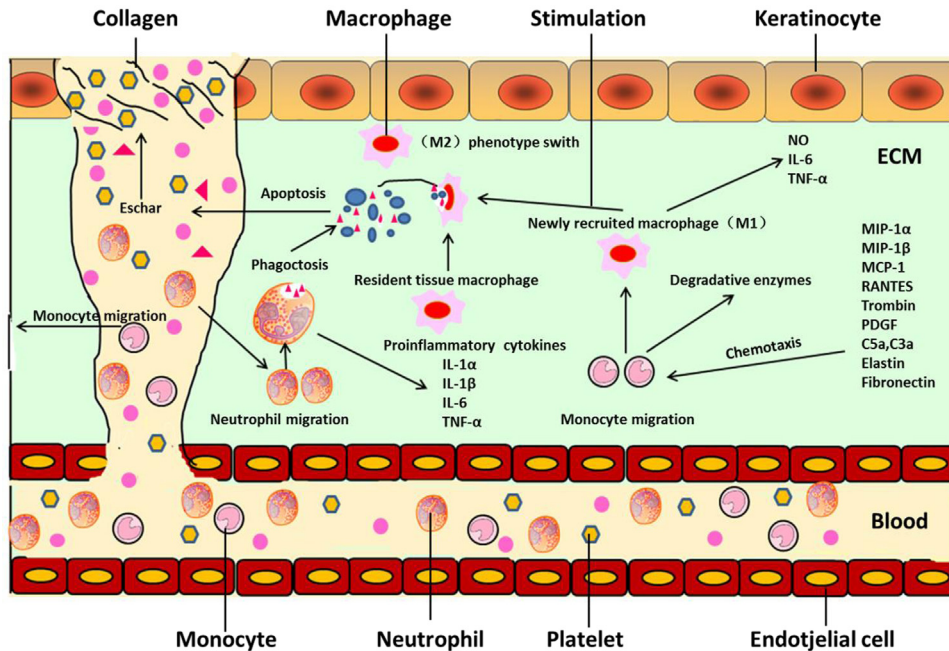


FIGURE 31.3 Inflammatory response phase. In the inflammatory stage, after migration, monocytes differentiate into macrophages. These macrophages in the wound bed can display different functional phenotypes, which can roughly be divided into two groups: M1 (classically activated) and M2 (alternatively activated) macrophages. M1 macrophages participate in proinflammatory responses and play a central role in host defense against bacterial and viral infections. Common proinflammatory cytokines include IL-6, TNF- α and interleukin-1 β (IL-1 β), while M2 macrophages are associated with antiinflammatory reactions, tissue remodeling, and fibrosis. In addition, macrophages probably include phagocytose debris and dead neutrophils. *IL-1 β* , Interleukin-1 β ; *IL-6*, interleukin-6; *TNF- α* , tumor necrosis factor alpha.

and monocytes concentrate in the inflammatory site over the endothelial cells of the blood vessel wall, thus triggering an inflammatory response [20]. Macrophages also play an important role in tissue repair and remodeling in the process of wound repair. For example, Chen et al. developed a multifunctional hybrid hydrogel that can significantly enhance the chronic wound-healing process of an infectious full-thickness skin defect by macrophage polarization and granulation tissue formation [21]. Macrophages can be polarized to early M1 macrophages and later M2 macrophages. M1 macrophages participate in proinflammatory responses and play a central role in host defense against bacterial and viral infections. Common proinflammatory cytokines include interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1 β). On the contrary, M2 macrophages are associated with antiinflammatory reactions, tissue remodeling, and fibrosis. Common antiinflammatory cytokines include interleukin-10 (IL-10) and interleukin-13 (IL-13) [22]. The dysfunction or imbalance of the macrophage phenotype affects healing and is

considered to be the underlying cause of inflammatory diseases of the skin. In the early stages of inflammation, the M1 phenotype is significantly increased at the site of injury. In the later stages of inflammation, the polarization of the macrophage population can become the M2 phenotype. M2 macrophages facilitate the regeneration of the skin towards the implantation site. If macrophages are not to be the polarization of the macrophage (M2 macrophages), the inflammation phase will lead to fibrosis and result in compromised healing [23,24].

The complement system which is an early, upstream recognition system, also triggers other inflammatory systems. However, it acts more downstream in the inflammatory reaction. For implanted biomaterials, the skin will launch a series of wound healing mechanisms, and inflammation is an important content in the complex process as it restores the surrounding tissue through the wound healing and eliminate pathogenic bacteria. Cytokines and chemokines are important mediators of inflammatory networks. Cytokines such as IL-6, TNF- α , and IL-1 β are key mediators of acute inflammation response, while chemokines such as IL-8, MCP-1, and MIP-1 β attract immune cells and trigger cytokine secretion. The activation of the complement system is the first line of defense before the secretion of cytokines and chemokines. In addition, due to a variety of cytokines and oxygen-free radical release, the inflammatory cell migration will also destroy the endothelial monolayer, eventually leading to material implant failure. Therefore, the degree and duration of inflammatory reaction directly affect the stability and histocompatibility of biomaterials, and thus affect the effectiveness of biomaterials.

Furthermore, the characteristics of the biomaterial determines the degree of the inflammatory response. For example, stainless steel causes a less intense inflammatory response and copper is stronger. Other materials such as aluminum, titanium, polyethylene terephthalate, and polytetrafluoroethylene stimulate similar inflammatory responses [25]. Titanium and tantalum relates to the antiinflammatory properties of surface oxides [26,27]. Titanium dioxide inactivates reactive oxygen species that are integral to initiating and maintaining the inflammatory response [28,29].

31.3.2 The hypersensitivity responses induced by biomaterials on skin

Some of the biomaterials inserted into the surface of the skin can cause hypersensitivity responses. Generally, there are four common types of hypersensitivity responses [30]. So far, hypersensitivity reaction types I–III have not been found in biomaterials, but hypersensitivity reaction type IV which is named delayed hypersensitivity reaction and mediated by lymphocyte cell (TDTH cell) has been documented [27].

Metal hypersensitivity in general is a well-established phenomenon. Metals have been reported to cause hypersensitivity responses with exposure to in vivo, skin contact, and ingestion of metals. Common skin lesions perform as eczema, hives, redness, and itching [31]. Many studies have shown that hypersensitivity response type IV is the major immunologic mechanism that metallic biomaterials lead to specific responses. Type IV hypersensitivity for some metallic biomaterials have been described such as cobalt, nickel, stainless steel, chromium, beryllium, and chromium, while occasional responses have been reported in relation to titanium, tantalum, and vanadium [32]. The most common metal in humans is nickel.

Hypersensitivity responses to nickel are well documented, and are a dermatological manifestation of type IV hypersensitivity, followed by cobalt and chromium. Cross-sensitivity reactions between nickel and cobalt are most common. Tantalum and titanium are well tolerated by most patients, and allergic reactions are extremely rare [33,34]. Skin contact sensitivity from titanium is rare, but it has happened in pacemaker-dependent patients [35]. Tantalum and titanium are appropriate choices for skin implantation with exceptional mechanical and noninflammatory properties. Furthermore, reports of adverse reactions for tantalum and titanium are rare.

31.3.3 The stimuli responses induced by biomaterials on skin

There are many approaches to introduce efficient stimuli signaling to enhance or speed up the skin healing process when the biomaterial comes into contact with the skin. Biomaterial scaffolds are biodegradable and provide temporal structural support, and it can adjust the cell behaviors and promote the growth of new tissue. It promotes the colonization of the different cells recruited when the biomaterial is implanted into the skin, and the establishment of different biochemical and biophysical signaling can promote cell migration, proliferation, and differentiation. Similarly, cells from the host will be recruited through the circulation towards the wound. In this section, we review the reaction of biomaterials to skin from the characteristics of biomaterials themselves.

31.3.3.1 Biomaterial physical properties

As shown in many works, biomaterial physical properties such as surface topography, geometry, and roughness play an important role in protein adsorption and cell response. Surface nanotopography is an important contributor to cell signaling. Different nanoscale features such as ridges, grooves, steps, and cliffs are introduced to facilitate cell attachment generally [36,37]. For example, designing different polystyrene nanogroove patterns enhanced the closure of the wound and promoted cellular migration and adhesion [38]. The orientation and density of nanogrooves also affected fibroblast's migration during wound healing [37]. Furthermore, to increase the contact area of the cells, larger surface features are generally used which range between 10 and 100 nm [39]. In addition, biomaterial charge and surface hydrophilicity are instrumental in the immune response to biomaterials. For example, anodization of titanium surfaces, which causes the development of titanium dioxide nanotubes on the surface, has been shown to cause significantly less proinflammatory cytokine release [40]. The researchers demonstrated that hydrophilic modified sand-blasted, acid-etched (SLA) surfaces caused downregulation of ten proinflammatory genes when compared to SLA surfaces only [41]. Several studies have been shown that biomaterial's geometry, including size and shape, play crucial roles in in vivo biocompatibility and cell response [42,43]. For example, Salthouse et al. studied various medical-grade materials with differently shaped cross-sections, the results show that rods with circular cross-sections produced the least-extensive foreign-body response (FBR), compared to pentagonal and triangular cross-sections [44]. In addition, the researchers examined the size of spherical biomaterials' in vivo biocompatibility from 0.3 to 1.9 mm in

diameter in rodents. The results show that larger implanted spheres, with diameters of 1.5 mm and above, significantly suppressed the FBR [45].

Nanotechnology offers excellent tools for the introduction of nanotopographic signaling in biomaterials. For example, nanomaterials can be combined within a larger guiding template or scaffold by modifying the surface and three-dimensional (3D) structure at the nanoscale. Electrospinning introduced active nanotopography in a 2.5D manner for skin regeneration, and maybe the cheapest and most efficient technique to produce ECM-like scaffolds made of submicronic and nanometric polymeric fibers [46,47]. Dendrimer structures such as nanosized highly branched polymers have also been engineered in the form of nanofibers providing an improvement in the inflammatory properties in wound healing [48]. Melting electrospinning can place fibers over a micron in size to create controlled textured scaffolds, but only for low melting-temperature polymers [49].

Dimensionality is one of the main concerns nowadays in skin wound healing. Current *in vitro* analyses are mainly carried out in 2D cultures of homogeneous populations of cell monolayers. However, there are differences in adhesion, migration and cytoskeletal evolution, proliferation, maturation, and cell signaling. 3D environments offer promising potential in terms of signaling modulation and biomimetism. For example, 3D cell printing technology is capable of accurately patterning living cells at predefined spatial locations. It also contributes to the formation of intricate microenvironments and the replication of indigenous skin tissue organizations [50–52]. 3D biomaterial matrix enables long term, full thickness, immunocompetent human skin equivalents with nervous system components [53]. Skin explants contain numerous cell types, maintain the ECM and the 3D structure of the tissue, and can be relatively simple to obtain. However, many 3D cell and tissue models for skin also lack other vital components such as blood vessels, nerves, and glands. In addition, patient variability, sourcing, and donor health are worthy of attention [54].

Finally, electrical stimulation can accelerate the healing in skin tissue, as cell migration is essential for wound healing. Electric fields have recently been proposed as a feasible way to speed wound healing by increasing proliferation, guiding, and angiogenesis [55,56]. Electric fields also seem to stimulate the increase of the expression of several signals and receptors [57,58].

31.3.3.2 Bioactive strategies

Developments in tissue regeneration have mainly relied on the application of cells and GFs in combination with biomaterials, and bioactive factors have significant biological activity *in vivo* in wound repair and regeneration. Furthermore, based on the nature of the biomaterial itself and the sensitivity and instability of GF, the effectiveness and safety of bioactive factors have not been fully demonstrated. Therefore, for proper healing of the wound, it is important to control the release rate and profile and avoid initial release bursts [59]. Furthermore, natural barriers and elimination mechanisms should also be considered, such as the stratum corneum or exudation, as these can make it difficult to reach the minimum therapeutic contact time needed between GFs and cells in the wound bed. This necessitates high dosage and numerous applications during therapy [60]. Apart from the associated high costs, this solution can induce serious side effects, including tumorigenesis [61]. Therefore, there is a need to circumvent such drawbacks by adopting new

strategies and approaches for tissue repair. The fact that cells naturally produce and regulate these GFs, cytokines and other signals should be taken into account.

So far, many engineered GFs have been used in skin wound healing and tissue engineering, such as the delivery of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) combined with scaffolds. PDGF has significant benefit in treating chronic wounds [62], especially diabetic foot ulcers [63] and it can also increase healing in excision wounds as well as wound healing in rodents [64,65]. Encapsulated EGF in biomaterials has many roles in skin wounds. For example, it can increase inflammatory recovery, epidermal regeneration and wound contraction [66,67], enhance fibroblast proliferation and the closure of full-thickness diabetic wounds [68,69]. VEGF and PDGF also elicit a better healing response which enhances in vivo wound healing by promotion of angiogenesis for VEGF and tissue regeneration and remodeling for PDGF [70].

Gene therapy is also an excellent approach to the skin wound healing. For example, DNA plasmid is a good option to treat cutaneous wounds [71]. It increases newly formed and mature vasculature and enables fast regeneration of the dermis in full thickness burns [72]. RNAi therapy plays an important role in chronic skin wound healing, especially in diabetic murine wounds. It increases the expression of angiogenic regulators matrix and HIF1 α in diabetic mice, reduces in fibrous capsule thickness, and accelerates wound healing in diabetic mice [73,74]. RNAi therapy allows the silencing of some gene expression by precisely targeting overexpressed biomolecules or enzymes such as MMPs in the environment of a chronic wound [75]. In addition, nanofibers impregnated with nucleic acids enhanced tissue regeneration and minimized scar formation in diabetic and healthy wounds [71].

31.3.3.3 Biomaterial mechanical properties and stimulus signal

Biomaterial mechanical properties mainly include strength, elongation, elastic modulus, shape memory effect and superelasticity, friction and wear performance, fatigue strength, etc. Excellent mechanical properties are the essential requirement for soft tissue repair, including high toughness, good flexibility, and recoverability. Recently, hydrogels have been designed for skin tissue regeneration, due to the good mechanical properties matching soft tissue. For example, inspired by the mussel adhesion chemistry, Gan developed a contact-active antibacterial hydrogel by copolymerization of methacrylamide dopamine (MADA) and 2-(dimethylamino) ethyl methacrylate and formation of an interpenetrated network with quaternized chitosan. The hydrogel had a high cell affinity, toughness, and recoverability for skin tissue repair [76]. In addition, tough hydrogels, including interpenetrating network hydrogel, double-network hydrogel, and nanocomposite hydrogel also have been designed for tissue regeneration [77–79]; such as polyampholyte hydrogels which are synthesized with positively and negatively charged units. These relatively charged groups form high-density electrostatic interactions in the hydrogel network, which provide the polyamphoteric electrolyte hydrogel with good mechanical properties. Metal materials also have good mechanical properties. For example, medical titanium alloy has good biological properties in oral prosthodontics.

Mechanical stimulation of the skin can introduce different biochemical signaling in the microenvironment including passive and active stimulation. When skin accepts active

stimulation from biomaterial, the spatial conformation of ECM components changes, as well as cell interaction with their neighbors and the ECM. It will produce some of the signaling molecules and affect cell communication with each other, such as the monocyte chemoattractant protein-1 (MCP-1/CCL2) from chemotactic peptides. It is normally linked to glycosaminoglycans from the ECM and can be easily released to the media and detected by endothelial cells or leukocytes which can speed up inflammation and tissue healing. Chemokines can control the migration of specific leukocyte populations during inflammatory responses, hematopoiesis, and routine immune surveillance, Inflammatory chemokines can also stimulate further cellular activation, resulting in destructive processes such as lysosomal enzyme release, generation of toxic products from the respiratory burst, and apoptosis [80]. Regarding passive stimulation, it was suggested that the controlled stimuli of the mechanical stiffness of a scaffold could promote the expression and synthesis of the VEGF receptor 2 (VEGFR2) [81]. Passive forces can also make cells exert contractile forces and modify their microenvironment, and the other way around the scaffold mechanical properties and introduced signaling can modify cell behavior, functions, and tissue regeneration [82].

Fibroblasts are the most important cells in the process of ECM remodeling in skin, control of wound contraction, and scar formation. During healing, skin is subject to various mechanical and other stimuli such as compression, tension, torsion, shear stress, etc. These stimuli favor the expression of α -SMA involved in the cytoskeleton of fibroblasts as one of the agents leading to the contraction of the wound [83]. Fibroblasts can be subjected to low and high mechanical stress, and they adapt by modifying their phenotype, which do not produce ECM components and promote cell migration when exposed to low intensity force. There is a decrease of the extracellular signal-regulated kinases signaling and less expression of GF receptors. When fibroblasts suffered high intense mechanical forces which can produce collagen and other ECM components, the cytoskeleton is reorganized, leading to promoted cell migration and proliferation [11,84].

31.3.3.4 Metal ions and inorganic compounds

Some inorganic compounds and ions are also used in skin wound healing. Metal ions have a huge effect on the skin which are crucial as body metabolism catalysts, as well as forming the components of structural elements of proteins, enzymes, or transcription factors [85]. The distribution of metal ions is specific to the observed area of the skin. A short summary of the most common metal ions used in skin wound healing is shown in Table 31.1. Unfortunately, the distribution of other minority trace metals is not well defined [86,87].

Calcium, magnesium, zinc, and copper ions gradients are well known and contribute to the normal epidermal homeostasis. In the skin, extracellular calcium plays a vital role both in healthy skin maintenance and in the healing process [115]. Calcium is a very well-known second messenger in signal transduction. Many extracellular signals trigger intracellular reaction cascades that lead to the activation of different calcium channels from the plasma membrane, or from intracellular reservoirs inducing sudden spikes of calcium in the cytoplasm [11]. Calcium also acts as a primary signal, since extracellular calcium can bind to transmembrane receptors activating intracellular cascades that modulate cell behavior to help skin healing [116]. In the skin, calcium is crucial for the differentiation of

TABLE 31.1 Summary of the most common metal ions used in skin wound healing.

Metal ions	Effect	Reference
Ca^{2+}	• Improves the wound healing process	[81,86]
	• Promotes angiogenesis	[81]
	• Binds to transmembrane receptors activating intracellular cascades that modulate cell behavior	[86]
	• Increases the expression of IGF family	[88]
	• Promotes hemostasis	[88]
	• Stimulates the levels of migration and proliferation of epidermal cells	[86]
Mg^{2+}	• Has a distinct impact on the adhesive and migratory activities of many cell types	[89]
	• Promotes skin wounds healing	[90]
Zn^{2+}	• Stimulates ECM resorption through MMPs	[88]
	• Augments autodebridement and keratinocyte migration during wound repair	[91]
	• Increases angiogenesis	[91]
	• Enhances collagen synthesis	[92]
	• Helps cell membrane repair, cell proliferation, growth and immune system function	[93]
	• Promotes reepithelization and it is antibacterial	[94]
	• Has antiinflammatory properties	[94]
	• Enhances cell adhesion, cell proliferation and migration	[95]
	• Enhances healing and nerve regeneration	[95]
	• Increases ATP activity	[90,96]
Ag^{+}	• Has antiinflammatory properties	[100]
	• Aids healing in the sterile skin wound	[101]
	• Has multilevel bactericidal and antimicrobial effects	[101]
	• Mediates differential responses in some of liver and kidney functions during skin wound healing	[102–104]
	• DFU does not offer evidences of healing apart from the antimicrobial effect in diabetic foot ulcers	[105]
	• Downregulates TGF- β	[106]
		[88]
Fe^{3+}	• $\gamma\text{-Fe}_2\text{O}_3$ magnetic nanoparticles increase mechanical resistance and healing speed	[107]
	• Dinitrosyl-iron complexes with cysteine or glutathione accelerate skin wound healing in animals	[108]
	• Dinitrosyl iron complexes with thiol ligands promote skin wound healing in animals	[109]
	• Iron chelation results in increased VEGF and HIF 1- α and positive effect on angiogenesis	[110]
	• Zinc/iron solution enhance healing in acute partial thickness and second-degree burn wounds	[93]
Cu^{2+}	• Increases skin elasticity and healing	[88,90]
	• Possesses antimicrobial, antiinflammatory properties	[111]
	• Promotes angiogenesis	[111]
	• Has an antibacterial effect	[112]

(Continued)

TABLE 31.1 (Continued)

Metal ions	Effect	Reference
	• Promotes partial thickness wound healing • Augments the level of differentiation of MSCs	[113] [114]

ATP, Adenosine triphosphate; *DFU*, diabetic foot ulcers; *ECM*, extracellular matrix; *IGF*, insulin-like growth factor; *MSCs*, mesenchymal stem cells; *TGF-β*, transforming growth factor beta.

keratinocytes in the healthy epidermis, as a cofactor in the coagulation cascade, and is implicated in the healing process [86]. Release of calcium to the wound bed definitely improves healing [117,118]. In addition, it can promote angiogenesis and stimulate the levels of migration and proliferation of epidermal cells [81].

There are other metals involved in skin healing. Studies report that zinc plays a crucial role in skin healing as well [85]. It can not only promote epithelial regeneration, but also promote cell adhesion, proliferation, and migration. It also has antibacterial and antiinflammatory properties [96–98]. Silver also shows antibacterial and antiinflammatory properties. Up to date, silver probably has no competitor. Copper also has shown an angiogenic effect in skin healing, which not only increases skin elasticity and healing, but also the level of differentiation of MSCs [119].

Some inorganic compounds play a crucial role in skin healing [120]. For example, nitric oxide (NO) can be an important factor in the skin healing process and it can regulate deposition of ECM proteins, cell proliferation, and endothelial function [121]. There have been several studies showing the increased wound healing rate due to delivery of NO in a wound microenvironment [120,122]. Silicon can be used for dermal reconstruction. Studies have shown that silicon-based glasses combined with chitosan and silk fibroin were potentially angiogenic-enhancing for skin regeneration [123].

31.4 The role of scaffolding materials in skin tissue engineering

Scaffolds are the best materials that play a unique role in restoring, maintaining, and improving tissue function. The skin tissue engineering scaffold provides an environment for the adhesion, growth, proliferation, and degeneration of seed cells, and is an important component of artificial skin to guide tissue regeneration and control tissue structure. Exploring ideal scaffold materials is a hot topic in the field of skin tissue engineering, in recent years, in terms of the current research progress in skin tissue engineering scaffold materials at home and abroad. It is divided into three categories including natural materials, synthetic materials, and composite materials.

Natural biomaterials can promote cell adhesion, proliferation, and differentiation, due to their resemblance to the natural ECM, they are highly biocompatible and biodegradability, thus are best suited for skin cell growth. In addition, natural materials have wide sources, simple preparation, and low price. However, it also has some problems such as poor mechanical properties, uncertainty of antigenicity elimination, and an uncontrollable

degradation rate. The most common are proteins and polysaccharides. Natural materials are widely used in wound and burn dressings. For example, natural polymers can stimulate the healing by repairing the damaged tissue and promoting effective skin regeneration. Synthetic biomaterials carry with good biocompatibility, controllable degradation rate and excellent mechanical properties. Widely used as scaffold materials for fabric engineering, synthetic biomaterials including various types of nanomaterials, such as polyvinylpyrrolidone, polycaprolactone, poly-ethylene-glycol, poly lactic acid, etc., are good at enhancing the strength of the scaffold material; but it is poor for cell adhesion and the hydrophilicity of the material. Therefore, more efforts towards developing composite scaffolds for skin tissue growth are required. The combination of such materials resolves around issues such as biocompatibility, biodegradability, and mechanical strength [124].

31.5 Future perspectives

The skin is the largest organ in the human body and consists of three main parts, with unique cells and structures. These cells induce various immune responses to outside stimuli in a coordinated manner. The microenvironments in skin are highly complex, dynamic, and multifaceted; it is also still in its infancy to regulate skin regeneration and wound healing. Biomaterials are used as platforms to generate stimuli that can promote cell activity related to skin regeneration. However, biomaterials are foreign bodies to the skin, which will produce some immune response or rejection in the body. If biomaterials are to be successful, the response must be accepted by the host without harmful effects at least. Therefore, a better match should be established between cell activity and biomaterials. At present, researchers pay more attention to skin personalized therapies, ignoring many problems. More secure and effective smart biomaterials need to be explored, and the mechanisms involved in skin healing require further studies.

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